00040.030240 PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	:	Examiner: Taofiq A. Solola
GERHARD HOEFLE, ET AL.	)	
	:	Group Art Unit: 1625
Application No.: 09/313,524	)	
	:	Confirmation No.: 4030
Filed: May 17, 1999	)	
	:	
For: EPOTHILONES C, D, E, AND F, PREPARATION AND COMPOSITIONS	)	
	:	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.131

Sir:

Pursuant to the provisions of MPEP 715, Applicants hereby aver as follows

1. I, Gerhard Hoefle, am a named inventor of the above-identified application.

2. I actually reduced to practice various species within the scope of the claims, or supervised the actual reduction to practice various species within the scope of the claims, before September 26, 1996. Copies of various laboratory notebook pages confirming this are attached. Dates on the attached photocopies are redacted, but as to paragraphs 1- 59, they are all prior to September 26, 1996, with the balance at least occurring prior to filing, as developed in the course of Interference No. 105,298. For the Examiner's convenience, an initial table is provided as a summary sheet (labeled

"Evidence for the discovery of epothilones C and D from...") as well. Note too that the laboratory notebook pages have been labeled "Exhibit 3-1, 3-2, etc." by me simply for easy reference herein.

3. Dr. Klaus Gerth, a microbiologist at Gesellschaft für Biotechnologischie Forschung mbH ("GBF"), instructed Carmen Fischer, a laboratory technician in the Chemistry Group at GBF, to commence cultivation and screening using a culture sample of *Sorangium cellulosum*, Soce1198 45/30.

4. Accordingly, Ms. Fischer took a sample of this Soce1198 45/30 strain, and inoculated it in heso; she recorded her work on a Sample Table, Exhibit 3-1, and in particular, she recorded the growth of this strain to be "good." Id.

5. Likewise, Ms. Fischer took a second sample of this Soce1198 45/30 strain, inoculated it in Probion (this is a protein supplied by, at that time, Hoechst AG as nutrient component), and took the resultant product and used it to inoculate a 250 ml shaking flask with medium S (S is the lab term for a culture medium used for the screening of Sorangium strains). Id.

6. Ms. Fischer used that material in turn to inoculate three 250 ml flasks with medium S, which she recorded was harvested. Exhibits 3-1 and 3-2.

7. Ms. Fischer recorded that two ("second" is a misinterpretation of "2 K." in the Eglish translation) of the three 250 ml flasks were "good." Exhibit 3-1.

8. The product harvested by Ms. Fischer was then given to Dr. Gerth, who proceeded to conduct an HPLC-UV absorbance analysis on the product – this analysis

was a "production check"; i.e., to check whether the strain was producing the desired materials.

9. In particular, Dr. Gerth injected the product onto an HPLC column and ran a UV absorbance analysis on the eluent, and the resultant spectrum is found in Exhibit 3-3.

10. On the spectrum found in Exhibit 3-3, Dr. Gerth wrote "Epo A" above the UV absorbance peak for the material eluted at 13.282 minutes, and wrote "Epo B" above the absorbance peak for the material eluted at the 14.584 minutes. Id. at page 1.

11. Dr. Gerth was able to identify these materials as epothilone A and epothilone B based upon his previous work with these materials.

12. His identification was also confirmed by the two UV peaks observed at the 13.282 and 14.584 time slices, characteristic of the presence of the thiazole side-chain (only thiazoles with the adjacent double bond show this peak pattern) found in epothilones A and B. See id. at page 4.

13. Additionally, Dr. Gerth noted at the same day the presence of an additional material or materials, which he recorded as "epo unbekannt" (meaning "unknown epothilones") on the spectrum. Id. at page 1.

14. Dr. Gerth identified this material in this manner because it also exhibited the characteristic UV bands of epothilone A and epothilone B, but plainly was not those materials, since it eluted at a different time. [See id. at page 6.]

15. C. Fischer summarized these results on the Sample Table (Exhibit 3-1), where she recorded the names "epothilon A," "epothilon B," and "epo. Unbekannt" adjacent their respective peaks.

16. Ms. Fischer provided Mr. Steinmetz with a 20 microliter (erroneously "ml" in the english translation) into sample of So ce1198 45/30 in a high pressure liquid chromatography ("HPLC") tube for analysis. Exhibit 3-4.

17. Mr. Steinmetz in turn gave the sample to Ms. Antje Ritter, a laboratory technician who also worked in the Chemistry group, and asked her to analyze the sample of Soce1198 using HPLC/UV and on-line mass spectrometry ("MS").

18. Ms. Ritter conducted the requested HPLC-UV analysis. Exhibit 3-5. In the same HPLC run she also conducted the MS analysis on the sample. Id.

19. The same day that the HPLC/UV/MS analysis was conducted, Ms. Pohlan reviewed the print-outs from the HPLC/UV/MS analysis and wrote down "Epo A" and "Epo B" next to the peaks in the chromatogram (page 1) and next to their corresponding UV spectra (pages 2 and 3); she additionally wrote down "Epo neu" ("new epothilones") next to the two peaks in the chromatogram as well as UV spectra corresponding to the newly identified, but as yet unisolated and uncharacterized, materials. Id.

20. Mr. Steinmetz reviewed the results of the ESI-MS analysis, and found that the two new materials showed protonated molecular ion ($M+H^+$) of 478.2 and

492.5 respectively, as compared to those of epothilone A, 494.4, and epothilone B, 508.6. Exhibit 3-5.

21. Mr. Steinmetz therefore realized that the new materials each differed from epothilones A and B by the atomic mass 16, which is the mass of an oxygen atom.

22. In addition, Mr. Steinmetz reviewed with Ms. Pohlan the HPLC/UV/MS results, and noted that the new materials, which exhibited the characteristic UV bands of epothilones, each had an atomic mass 16 less than epothilones A and B; accordingly, Ms. Pohlan then recorded "Epo neu" (meaning "new epothilone") adjacent the UV absorbance curves taken for the 29.25 and 30.57 time slices. Exhibit 3-5.

23. Mr. Steinmetz discussed with Ms. Pohlan the 16 amu mass difference, and she recorded below the "Epo new" entries the equation "mz = -16," meaning that these new materials differed in mass from epothilones A and B by 16, respectively. Exhibit 3-5, at page 3.

24. Upon analyzing the data, Dr. Hoefle and Mr. Steinmetz concluded that the new materials had the same structures as epothilones A and B, except that they were missing an oxygen atom, presumably the epoxide group (one oxygen atom, atomic mass 16).

25. A departmental meeting was attended by Drs. Hoefle, Gerth, Reichenbach, Mr. Steinmetz and others, and was recorded in Meeting Minutes prepared by Dr. Reichenbach. Exhibit 3-6.

26. At the departmental meeting, Dr. Gerth reported his finding that the Sorangium cellulosum Soce 1198 strain produced epothilones A and B, and additionally produced small quantities of two unknown compounds exhibiting the characteristic ultraviolet (UV) spectrum of epothilone A and B, and that the new compounds were more lipophilic than epothilones A and B.

27. The "two peaks" referred to by Dr. Gerth at the departmental meeting were the thiazole double peaks exhibited by the UV absorbance spectrum.

28. In the Minutes of the meeting Dr. Höfle wrote:

> According to HPLC/MS studies carried out by Herr Steinmetz, the substance is composed of homologues (Δ14) possessing one oxygen atom less than epothilone A and B. There were ca. 1-2 mg of the new epothilones present in the shaken culture.

Exhibit 3-6 (English translation).

29. Dr. Höfle's reference to "homologues" was intended to indicate that the new compounds had a similar structure to the known compounds epothilone A and epothilone B, except for the absence of an oxygen atom. Id.

30. Dr Höfle recorded that the next step was to isolate the new compounds individually, or as a mixture. Id.

31. Ms. Pohlan received from Dr. Gerth a methanol extract of adsorber resin collected by Dr. Gerth from the shaking flasks in the secreening, which was labeled "So ce1198-45/30, Screening." Exhibit 3-7.

32. Ms. Pohlan evaporated the MeOH off, and recorded that she had obtained 198 mg ("g", gram is an error in the English translation) of material. On the same day she run an analytical tlc (on the left hand side of Exhibit 3-7) comparing the extract with authentic epothilone A. Exhibit 3-7.

33. Ms. Pohlan fractionated the material using a Sephadex LH-20 column 1.5 cm in diameter and 70 cm long, and obtained six fractions, which she recorded as "LH-1" through "LH-6," respectively. Exhibit 3-7.

34. Ms. Pohlan spotted these fractions onto different positions of a thin layer plate, and she recorded that the developed chromatogram showed spots in fraction "LH-2," which indicated that the epothilones A and B as well as the new compounds would be present in that fraction. Exhibit 3-7.

35. Ms. Pohlan conducted an analytical check on fraction LH-2 by subjecting a small sample to reverse phase HPLC separation and UV detection. Exhibit 3-9.

36. The UV absorption trace of the analytical check on fraction LH-2 displayed in the 2.43 time slice the characteristic UV spectrum of epothilones which confirmed the presence of epothilone A and epothilone B. Exhibit 3-9.

37. The UV absorption trace of the analytical check on fraction LH-2 further displayed in the 4.26 and 5.04 time slices the characteristic UV spectrum of epothilones, thus confirming that this sample also contained the new compounds. Exhibit 3-9.

38. Ms. Pohlan recorded in her notebook that she had conducted a reverse phase ("RP") chromatographic fractionation on sample LH-2, using a Nucleosil 100 column (20 x 250 mm) and a solvent consisting of 73 parts methanol and 27 parts water. Exhibit 3-10.

39. Ms. Pohlan also obtained a UV absorbance trace of the eluent, and on that trace she noted that fractions 35 to 37 exhibited the peaks corresponding to epothilone A and epothilone B, which she labeled "epo A" and "epo B" respectively on the trace. Exhibit 3-11.

40. However, Ms. Pohlan also noted a UV absorption peak spanning fractions [46 and 47], which she labeled "RP-1" on the trace, and another UV absorption peak spanning fractions 50 and 51, which she labeled "RP-2" on the trace.

41. These UV absorbance peaks were believed to have been produced by the new compounds, and thus eluted fractions 46 and 47 were believed to contain one of the new compounds, and eluted fractions 50 and 51 were believed to contain the other of the new compounds.

42. Ms. Pohlan used a thin layer chromatograph technique to analyze a small amount of each of the eluents corresponding to peaks RP-1 and RP-2, and she recorded that the resultant spots had a violet color, which is the color that epothilones A and B were known to develope after spraying with vanillin/sulfuric acid. Exhibit 3-10.

43. Dr. Hoefle instructed Ms. Pohlan to submit the fraction corresponding to peak RP-2 for NMR analysis. Id.

44. Ms. Pohlan submitted the sample with an NMR Request Form, requesting a proton analysis (standard spectrum and COSY, 1D and 2D, respectively); the NMR analyses were conducted on the same day, and the resultant standard spectrum was given Spectrum no. 2550. Exhibit 3-12.

45. Spectrum no. 2550 for sample RP-2 was given to Dr. Hoefle; he reviewed it and found it to have characteristics of epothilone B, such as the five methyl singlets in the range of 1 to 2.7 ppm, and an olefinic singlet around 6.6 ppm. Exhibit 3-12.

46. However, Dr. Hoefle noted the presence in the NMR spectrum for sample RP-2 of a singlet at about 1.7 ppm; if the substance were epothilone B, this singlet would have been present at about the 1.2 ppm position.

47. Thus the singlet location was shifted, relative to epothilone B.

48. Given the previously recognized mass difference of 16, which is the weight of an atom of oxygen, Dr. Hoefle attributed the singlet shift in the RP-2 sample, relative to epothilone B, to the presence of a double bond, which had replaced the epoxide group.

49. After reviewing the NMR print-out, Dr. Hoefle sketched on it the CH_3 structure that he attributed to the singlet on the NMR print-out. See Exhibit 3-12.

50. Dr. Hoefle also drew a more complete picture of the molecular structure of the RP-2 material on the COSY NMR spectrum, which shows in addition to the structure of the -CH=CHCH$_3$- group that replaced the epoxide group, the surounding partial structures of epothilone. Exhibit 3-12.

51. The RP-2 eluent material, which Dr. Hoefle structurally characterized in the manner explained above, is the material that Dr. Hoefle and Mr. Steinmetz named "epothilone D."

52. As to the eluent material identified as RP-1, Dr. Hoefle noticed from the chromatograph (Exhibit 3-11) that it sat on a broader peak, which would suggest that it was mixed with other material.

53. Accordingly, Dr. Hoefle directed that the material RP-1 be further purified, and Ms. Pohlan subjected sample RP-1 to separation on silica gel plate. Exhibit 3-13.

54. Ms. Pohlan then used a thin layer chromatographic technique to analyze the resultant RP-1/DC, (DC means purified by thin-layer chromatography), and recorded that it exhibited a single band only, indicating a purified product. Id.

55. The next day, Ms. Pohlan submitted the purified sample of RP-1 for NMR proton analysis, and the resultant NMR was given Spectrum no. 2630. Exhibit 3-15.

56. Ms. Pohlan showed the NMR print-out for sample RP-1 to Dr. Hoefle, and he was immediately able to characterize the structure of the material, which he drew on the right-hand side of the NMR print-out. Exhibit 3-15.

57. The RP-1 eluent material, which Dr. Hoefle structurally characterized in the manner explained above, is the material that Dr. Hoefle and Mr. Steinmetz named "epothilone C."

58. A departmental meeting was held which was attended by a number of people, including Drs. Hoefle, Reichenbach, Sasse and Mr. Steinmetz and was recorded in Meeting Minutes prepared by Dr. Reichenbach. Exhibit 3-16.

59. At the meeting, Dr. Gerth, Mr. Steinmetz and Dr. Sasse reported to the attendees the following:

> The strains So ce1198, So ce1275 and So ce1294 form two new epothilones as well as epthilone, but with the epoxide missing (Gerth, Steinmetz). They had considerably reduced action, but were not abolished: the IC50 for L929 cells was 150 ng/ml for RP1 (from So ce1198), and 100 ng/ml for RP2. Noticeable effect on Tubulin could be detected in cell cultures. (Sasse) Perhaps patenting is possible?

Exhibit 3-16 (English translation).

60. Notably, the above, initial work was completed using strains So ce1198, 1275 and 1294. Further isolation work of epothilone C and D was then conducted using a variant or mutant strain of Sorangium cellulosum, So ce90. The wild version of So ce90 had previously been deposited with the German Collection for Microorganisms ("Deutsche Sammlung von Mikroorganismen") as DSM 6773.

61. A number of cultures were prepared from DSM 6773. These cultures, which were called "clones" generally do not have the same population mixture or production profile as DSM 6773.

62. In particular, So ce90 A3, which based on earlier work was known to be a good producer of epothilone A and epothilone B, was used for this further isolation work since its production profile was similar to that of So ce1198. Ms. Fischer recorded

the preparation of cultures medium for 15 L and 150 L fermentors (having working volumes of 10 L and 100 L respectively). Exhibits 4-10 to 4-12. The details and monitoring of these fermentions were also recorded, including a description of the fermentation medium used. Exhibits 4-13 to 4-26.

63. The product of these fermentations was then used to charge a 750 L fermenter. The details and monitoring of this fermentation was also recorded, including a description of the fermentation medium used. Exhibit 4-27 to 4-34.

64. The harvest of this fermentation was then undertaken by recovering the XAD absorber resin from the 750 L fermenter, filtering the absorber resin, (Exhibit 4-35 to 4-36); eluting the absorber resin with methanol, (Exhibit 4-37 to 4-38); concentrating the eluent by evaporation to a 20 L concentrate, (Exhibit 4-39 to 4-40); performing an ethyl acetate extraction, (Exhibit 4-41 to 4-42); and then subjecting the extract to rotary evaporation to yield crude extract, (Exhibit 4-43 to 4-44).

65. The crude extract was next tested for the presence of epothilone A, B, C and D. See Exhibits 4-45 to 4-49, particularly the mass spectrometer results shown in Exhibit 4-49, which depict the peaks indicative of the presence of these species.

66. The crude extract was then dried, distributed between methanol and heptane (the heptane was discarded), (Exhibit 4-50 to 4-51); and passed through a Sephadex LH20 chromatographic column, (Exhibit 4-52). Fractions were collected and utilized for further analysis. Exhibits 4-53 to 4-57. Again, the mass spectrometer results shown in Exhibit 4-57 exhibited peaks indicating epothilone A, B, C and D were present.

67. A reverse phase chromatography was next performed using fractions 6-12. Exhibits 4-58 to 4-60. A UV absorbance analysis indicated that fractions 8-12 contained epothilone A and B. Exhibits 4-61 to 4-63. Fraction 24, on the other hand, was subject to HPLC MS analysis, and was found to exhibit a peak indicating the presence of epothilone C. Exhibit 4-64. Similarly, fraction 28 was found to exhibit a peak indicating the presence of epothilone D. Exhibit 4-66. Mass spectrometer analyses confirmed these results. Exhibits 4-65, 4-67.

68. The epothilone C and D in the fractions referenced above were next purified using reverse phase RP-18 chromatography, and then analyzed. Exhibits 4-68 to 4-75. In particular, fraction RP-1 was subjected to UV absorbance analysis and exhibited a clean peak, indicating that it contained pure epothilone D. Exhibit 4-74. Fraction RP-2, which was epothilone C, was subject to TLC analysis. That analysis showed it to be free of trace contaminants. Exhibit 4-71.

69. There followed an NMR analysis of fraction RP-2, which confirmed the peaks as those of epothilone C. Exhibit 4-78 to 4-91. An NMR analysis was also conducted of fraction RP-1, which confirmed the peaks as those of epothilone D. Exhibit 4-92 to 4-105.

70. The data from the tests run with the So ce90 A3 clone were then used to prepare Example 1 in the subject application. Thus, the data reported in Example 1 were not generated with DSM 6773, and DSM 6773 was erroneously listed in the application as the starting material.

71. To demonstrate that wild strain DSM 6773 produces epothilones C and D, the strain DSM 6773 was ordered in 2005 and the production process as reported in the subject application was followed to generate and isolate epothilones C and D. These experiments are described at page 5 of the accompanying document, titled "Reply to the Opposition Statement against EP-B-1186606." (For completeness of the record, "epothilones A and B" at page 6, line 6 therein should read --epothilones C and D--.) The experiment produced 1.4 mg epothilone C and 0.5 mg epothilone D as reported in the attached Reply.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Gerhard Hoefle

Date: June 10, 2008

FCHS_WS 2196910_1.DOC

Evidence for the discovery of epothilones C and D from *Sorangium cellulosum* at the GBF in early [redacted]

No.	Date of document	Type of document	Operation, Results
1.	[redacted]	Screening record	*Sorangium cellulosum*, strain So ce1198 produces two new epothilones
2.	[redacted]	Screening record	HPLC sample preparation
3.	[redacted]	HPLC/DAD chromatogram	Two minor components identified as new lipophilic epothilones
4.	[redacted]	Sampling record	Sample given to H. Steinmetz for HPLC/MS analysis
5.	[redacted]	HPLC/MS chromatogram	The new epothilones contain one oxygen less than epothilones A and B
6.	[redacted]	Record of project meeting No. 230	Two new epothilone homologues with one oxygen less than epothilones A and B
7.	[redacted]	Isolation record	TLC and Sephadex LH 20 chromatography, enriched fraction
8.	[redacted]	LH 20 chromatogram	Separation of crude extract
9.	[redacted]	Analytical HPLC	The two new epothilones localised (X)
10.	[redacted]	Separation record	Fractions RP1 (0.7 mg) and RP2 (1.0 mg) isolated
11.	[redacted]	RP18 chromatogram	Separate peaks for RP1 and RP2
12.	[redacted]	NMR Spectra	^{1}H and COSY spectra prove that RP2 is an epothilone with a methyl substituted 12,13 double bond later named epothilone D
13.	[redacted]	TLC	Purification of fraction RP1 to give RP1/DC
14.	[redacted]	Flow diagram	Origin of the two new epothilones (C and D)
15.	[redacted]	NMR spectra	^{1}H spectrum proves that RP1/DC is an epothilone with a 12,13 double bond later named epothilone C
16.	[redacted]	Record of project meeting No. 231	The two new epothilones show reduced cytotoxicity and tubulin activity

Exhibit 3-1

EXHIBIT A

DECLARATION OF TRANSLATOR

I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

do hereby avow and declare that I am conversant with the English and German languages and am a competent translator of German into English. I declare further that to the best of my knowledge and belief the following is a true and correct translation prepared and reviewed by me of the document in the German language attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of U.S. Patent Application Serial No. 09/313,524 or any patent issued thereon.

R Richards

Date: 5. August 2003

03/4169

1

Table 1

Strain: *1198 – 45/30*	Aim: *Screening*	**Growth**	**Liquid culture**	**Growth**
Received on: [redacted]	Inoculated on heso: [redacted]	*good*	Inoculated in 50 ml heso: [redacted]	*good*
Preserved:	Inoculated on propion: [redacted]		Inoculated in 50 ml propion (German assumed to be misspelled):	
			Inoculated in 250 ml S : [redacted]	*moderate*
			Inoculated in 3 x 250 ml am (formate?): [redacted]	
				Harvest: *30.4*
			Medium 360: *1st flask very dark,*	*2nd flask good*
			Medium propion (German assumed to be misspelled):	
			Medium heso:	
Screening on:				
	Inhibition dil. step:			Substances
E.coli			**HPLC**	
Micrococcus			Method screen 1	
Staph				
Nocardia				
Mucor				
Hansenula			Method screen 2	
Candida			*13.282 HP*	*Epothilone A*
Schizo			*14.584 NP*	*Epothilone B*
Rhodotorula			*20.204 + 21.458 NP*	*Unknown epo*
			Special comments:	

No b. scr. sample was prepared by Herr Steinmetz!

Stamm: 1198 - 45/30	Ziel: Screening	Wachstum	Flüssigkultur	Wachstum
Erhalten am: [redacted]	Überimpft auf Heso: [redacted]	gut	Überimpft in 50 ml Heso: [redacted]	gut
Konserviert:	Überimpft auf Propion: [redacted]		Überimpft in 50 ml Probion:	
			" in 250 ml S: [redacted]	mäßig
			Überimpft in 3 x 250 ml am: [redacted]	
				Ernte: 30.4.
			Medium 360: 1 Kolben sehr dunkel	2K. gut
			Medium Probion:	
			Medium Heso:	
Screening am: [illegible]				Substanzen
	Hemmung Verd. Stufe:		HPLC	
E. coli			Methode Screen.1	
Micrococcus			13.282 HP	Epothilon A
Staph			14.584 NP	Epothilon B
Nocardia			20.204 + 21.458 NP	Epo. unbekannt
Mucor			Methode Screen.2	
Hansenula				
Candida				
Schizo				
Rhodotorula				
			Besonderheiten:	

kein B. Sc. vor
Probe wurde
Hr. Skiuwelt,
aufgearbeitet.

Exhibit 3-2

EXHIBIT

DECLARATION OF TRANSLATOR

I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

do hereby avow and declare that I am conversant with the English and German languages and am a competent translator of German into English. I declare further that to the best of my knowledge and belief the following is a true and correct translation prepared and reviewed by me of the document in the German language attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of U.S. Patent Application Serial No. 09/313,524 or any patent issued thereon.

R Richards

Date: 6. August 2003

[page of handwritten notes]

Monda[redacted]

- Sample from F100 fetched, microscope inspection → OK
  Can be inoculated
- Protocol for fermentation comp. 55 taken to Herr Schüler,
  Flask order to Frau Heiber.
- Medium + XAD from Ratjadon – fermentation taken to Herr Ebert
- Strain cultures inoculated + for fermentation
- Antifoam flask + alkali flask autoclaved, antifoam sterilised in drying cupboard →
  transferred/decanted (sterile)
- Sample from F900 checked → OK
- 1 litre P medium boiled ; E medium → thorax treatment → autoclaved.

2

100% gluc - 0% H_2O

35% gluc

35 ml gluc 65 ml H_2O = 100 ml

3 times amount added — 300 µl / 50 ml P medium

==========

Tue[redacted]

- Screening - strains : Harvest!
  1198 – 45/30 , 1230 , 1233 , 1235 :
- 7.30 - sample removed , preparation as for HPLC →
  take methanol flask to Herr Steinmetz →
  carries out analysis
- check other fermentation protocols
- HPLC of Soce90 clone (medium with skimmed milk from KS)
- 2 new screening - strains prepared in 10 ml H medium : Soce1266 + 1257
- For fermentor : Soce360A1 further inoculated / 6 flasks available
  : Soce 1149 " " / 3 flasks available

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Thu[redacted]

- Fermentor sample : HPLC – preparation (Herr Steinmetz)
- Screening – strains -- analysis (1198 – 45/30 ; 1227; 1230; 1233; 1235; 1251)
- Protocol 44 – first evaluation
- Fermentor protocols : sterility check , further inoculation
- Mon, Soce 1149 3 flasks, further inoculation → 6 flasks
- Fri, Soce 360A1 1,5 litres in inoculation flask → inoculation deadline 10.15

[redacted]

- Probe vom F 100 geholt, mikroskopiert → i. O. kann überimpft werden
- Protokoll für Fermentation Komp. 55 zu Hr. Schüler gebracht
  Flaschenbestellung zu Fr. Heiber
- Medium + XAD von Zafjadon-Fermentation zu Hr. Ebert gebracht
- Stammkulturen überimpft + für Fermentation
- Antischaum- + Laugenflasche autoklaviert, Antischaum im Trockenschrank sterilisiert → umgefüllt (steril)
- Probe aus F 900 kontrolliert → i. O.
- 1 l P-Medium gekocht, E-Med → Thoraxbehandlung ⟹ autoklaviert
- Medium für Komp. 55 - Fermentation abgerufen

100 % Glc — 8 % H_2O

35 % Glc

35 ml Glc 65 ml H_2O = 100 ml

2 fache Menge = 300 µl / 50 ml P-Med

[redacted]

- Screening-Stämme: Ernte!
  1198-45/30, 1227, 1230, 1233, 1235
- 7[32] Uhr - Probenentnahme, Aufbereitung wie zur HPLC
  das Methanol-Fläschchen zu Hr. Steinmetz bringen → führt die Analyse durch
- andere Fermentationsprotokolle nachsehen
- HPLC von Soce 90 Klon (Medium mit Magermilchpulver aus KS)
- 2 neue Screening-Stämme in 10 ml H-Medium angesetzt: Soce 1266 + 1257
- Für Fermenter: Soce 360 A1 weitergeimpft (6 Kolben vorhanden)
  " : Soce 1149 " (3 Kolben vorhanden)

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20 [redacted]

- Fermenterprobe: HPLC-Vorbereitung (Hr. Steinmetz)
- Screening-Stämme - Analyse (1198 45/30, 1227, 1230, 1233, 1235, 1251)
- Protokoll 44 → Auswertung
- Fermenterprotokolle: Sterilitätskontrolle, Weiterimpfung
  - Mo. Soce 1149 3 Kolben weiterimpfen → 6 Kolben
  - Fr., Soce 360 A1 1,5 l in Animpfflasche → Animpftermin 10°°

Exhibit 3-3

Epo unknown
Epo unbekannt

Data File C:\HPCHEM\1\DATA\000-0101.D Sample Name: 1198-45/30

Injection Date : 0[redacted] 13:38:30
Sample Name : 1198-45/30
Acq. Operator : Gerth

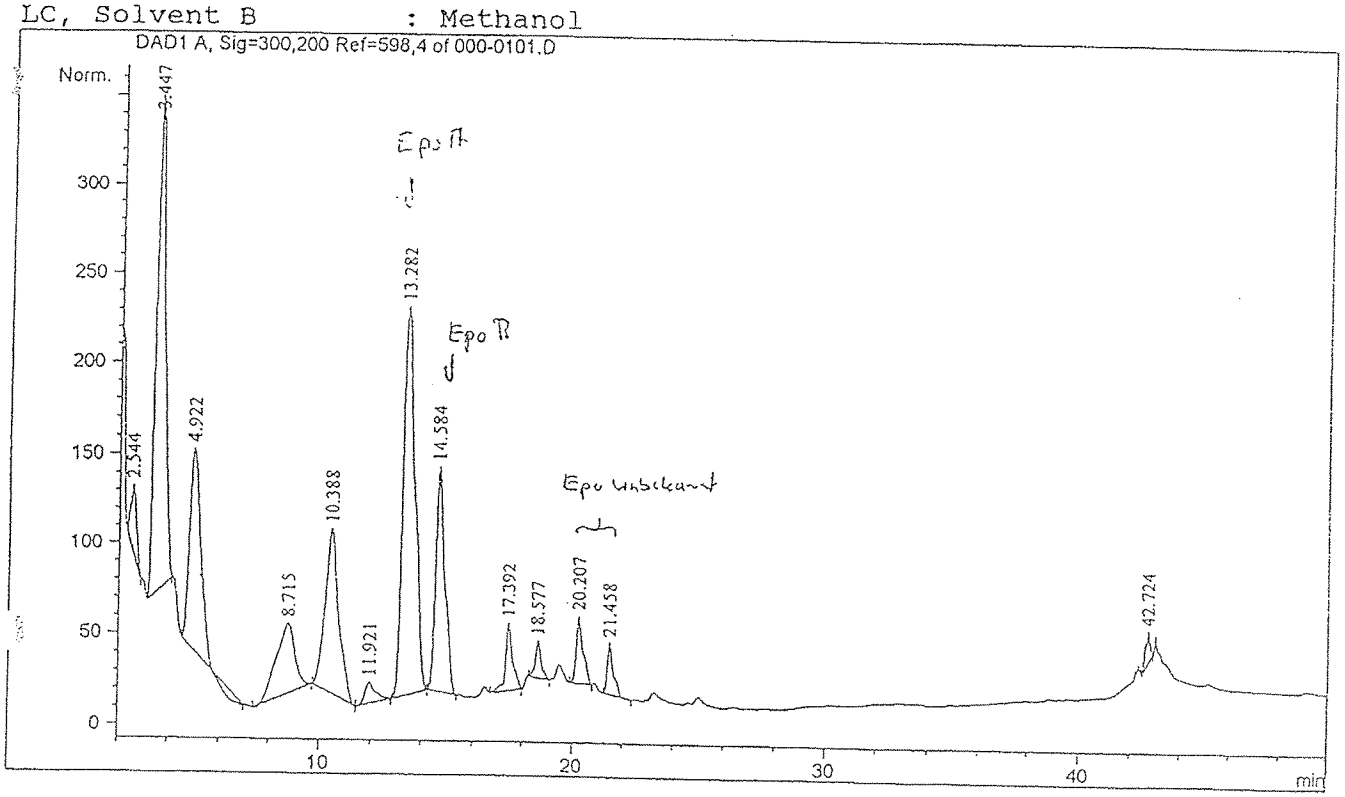
Seq. Line : 1
Vial : 0
Inj : 1
Inj Volume : 10 µl

Sequence File : C:\HPCHEM\1\SEQUENCE\DEF_LC.S
Method : C:\HPCHEM\1\METHODS\SCREEN1.M
Last changed : 0[redacted] 12:36:13 by Gerth
Screening 1 Methode

3

Instrument Conditions: At Start At Stop
Temperature: 39.8 39.8 °C
Pressure: 190.0 213.0 bar
Flow: 0.500 0.500 ml/min

Solvent Description :
LC, Solvent A : Wasser
LC, Solvent B : Methanol



Area Percent Report

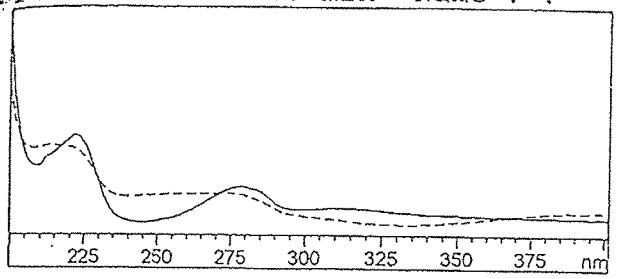
Sorted by Signal
Multiplier : 1.000000
Dilution : 1.000000

Signal 1: DAD1 A, Sig=300,200 Ref=598,4

Peak #	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	2.544	BB	0.208	580.48407	37.79762	2.0091
2	3.447	BB	0.380	6948.34668	277.56702	24.0491
3	4.922	BB	0.443	3222.51660	111.79380	11.1535

	RT [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.715	BB	0.689	1983.70227	39.05926	6.8658
2	10.388	BB	0.513	3327.00781	91.31287	11.5152
3	11.921	BB	0.400	344.29031	11.95683	1.1916
4	13.282	BB	0.466	6934.54688	215.95573	24.0013
5	14.584	BB	0.355	3090.42578	125.53922	10.6963
6	17.392	BB	0.289	759.20789	37.43296	2.6277
7	18.577	BB	0.231	320.88089	20.30108	1.1106
8	20.207	BB	0.265	701.43530	37.26771	2.4278
9	21.458	BB	0.228	453.19839	28.68863	1.5686
10	42.724	BB	0.213	226.30806	17.73971	0.7833
Totals :				28892.35156	1052.41248	

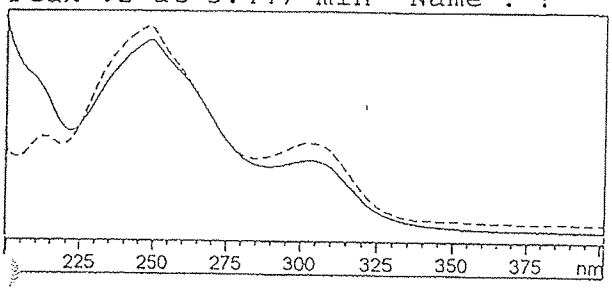
Peak :1 at 2.544 min Name : ?



-> The purity factor exceeds the thres

Purity factor : 796.557 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (2.545167
Spectra : 2 (Selection automatic, 3)

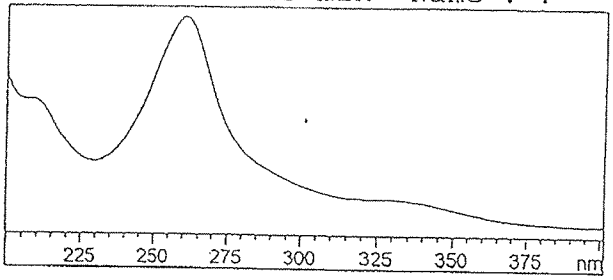
Peak :2 at 3.447 min Name : ?



-> The purity factor exceeds the thres

Purity factor : 842.491 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (3.444667
Spectra : 2 (Selection automatic, 3)

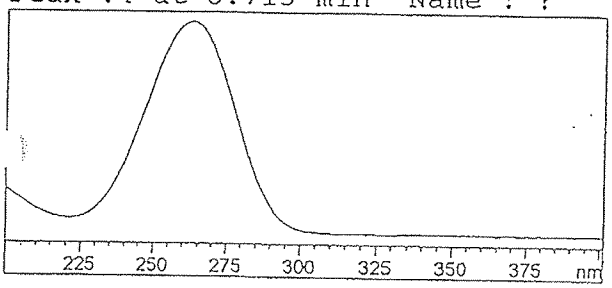
Peak :3 at 4.922 min Name : ?



-> Not enough data for purity calculat

Purity factor : Not available
Threshold :
Reference : Peak Apex (integrated) (4.925)
Spectra : 1 (Selection automatic, 3)

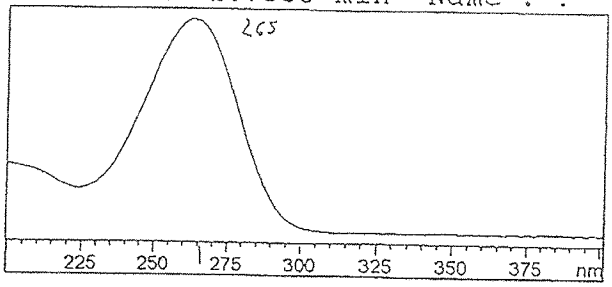
Peak :4 at 8.715 min Name : ?



-> Not enough data for purity calculat

Purity factor : Not available
Threshold :
Reference : Peak Apex (integrated) (8.719333
Spectra : 1 (Selection automatic, 3)

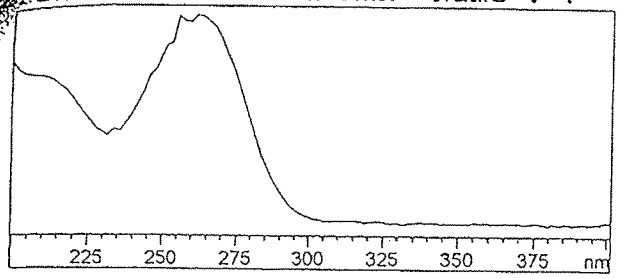
Peak :5 at 10.388 min Name : ?



-> Not enough data for purity calculat

Purity factor : Not available
Threshold :
Reference : Peak Apex (integrated) (10.38833
Spectra : 1 (Selection automatic, 3)

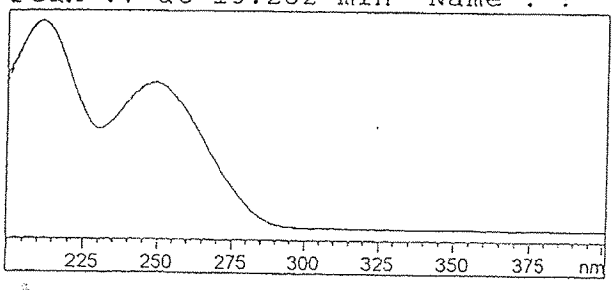
Peak :6 at 11.921 min Name : ?



-> Not enough data for purity calculat

Purity factor : Not available
Threshold :
Reference : Peak Apex (integrated) (11.91916
Spectra : 1 (Selection automatic, 3)

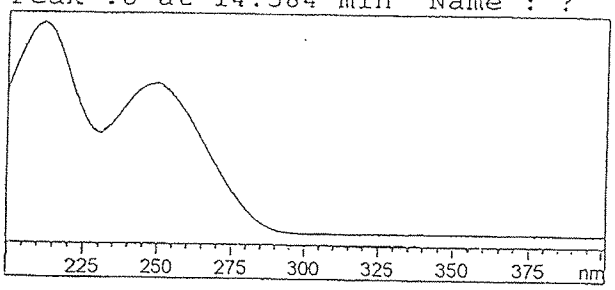
Peak :7 at 13.282 min Name : ?



-> The purity factor is within the thr

Purity factor : 999.671 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (13.28033
Spectra : 2 (Selection automatic, 3)
Warning : Spectral absorbances > 1000 mAU

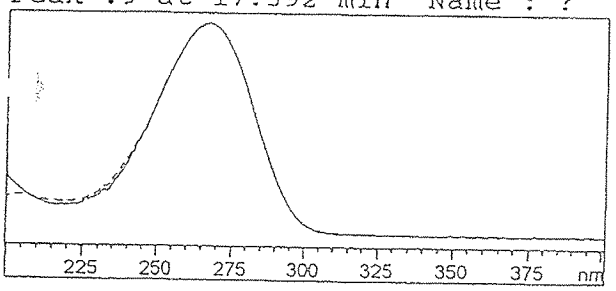
Peak :8 at 14.584 min Name : ?



-> The purity factor is within the thr

Purity factor : 999.896 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (14.5725)
Spectra : 2 (Selection automatic, 3)

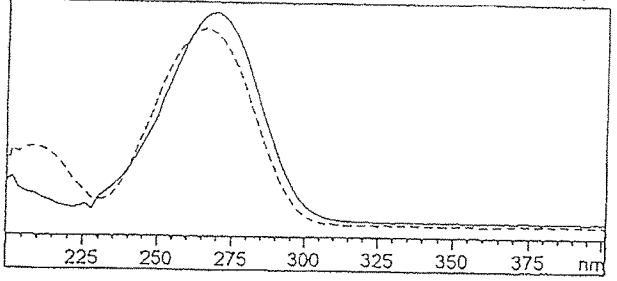
Peak :9 at 17.392 min Name : ?



-> The purity factor is within the thr

Purity factor : 997.584 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (17.38966
Spectra : 2 (Selection automatic, 3)

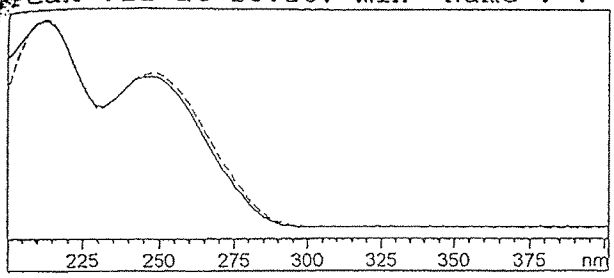
Peak :10 at 18.577 min Name : ?



-> The purity factor exceeds the thres

Purity factor : 922.261 (100% of spectra)
Threshold : 990 (Set by user)
Reference : Peak Apex (integrated) (18.57533
Spectra : 2 (Selection automatic, 3)

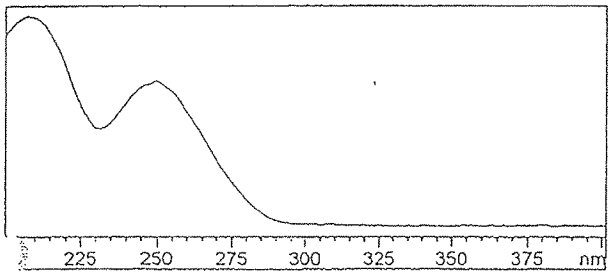
Peak :11 at 20.207 min Name : ?



-> The purity factor is within the thr

Purity factor	:	994.925 (100% of spectra)
Threshold	:	990 (Set by user)
Reference	:	Peak Apex (integrated) (20.2055)
Spectra	:	2 (Selection automatic, 3)

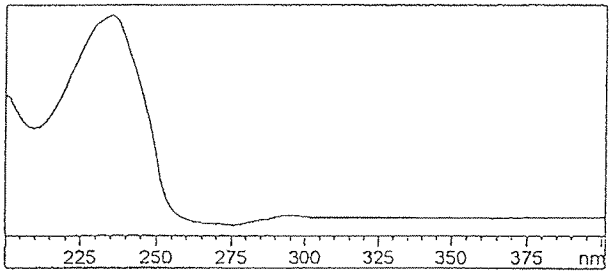
Peak :12 at 21.458 min Name : ?



-> Not enough data for purity calculat

Purity factor	:	Not available
Threshold	:	
Reference	:	Peak Apex (integrated) (21.45866
Spectra	:	1 (Selection automatic, 3)

Peak :13 at 42.724 min Name : ?



-> Not enough data for purity calculat

Purity factor	:	Not available
Threshold	:	
Reference	:	Peak Apex (integrated) (42.72333
Spectra	:	1 (Selection automatic, 3)

*** End of Report ***

Exhibit 3-4

EXHIBIT

DECLARATION OF TRANSLATOR

I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

do hereby avow and declare that I am conversant with the English and German languages and am a competent translator of German into English. I declare further that to the best of my knowledge and belief the following is a true and correct translation prepared and reviewed by me of the document in the German language attached hereto.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of U.S. Patent Application Serial No. 09/313,524 or any patent issued thereon.

R Richards

Date: 5. August 2003

[page of handwritten notes]

- Soce 660 Epo

H (only survivors inoculated) very good
(illegible comment in margin) H very good
H very good HPLC, Tues. 21.5

Tues. [redacted]

- HPLC of different samples or cultures
- New : prepare in liquid culture + plate :
  Soce 1241 sci.
  1198 – 45/30 several plates
  1199
  471 Epo
  523 Epo
  613 Epo

- Check Soce's on plate – mould?
- ~~Plates~~ from protocol 44 cleared away into cool room
- 20 ml Soce 1198 –45/30 given to Herr Steinmetz in HPLC tube for analysis

PROTOCOL 45 STILL HAS TO BE CARRIED OUT USING SOCE 1198 – 45/30 !

ALSO PROTOCOL 37 ! ALSO PROTOCOL 44 !

- Protocol 37 :

Boil 1.5 litres E medium

E medium for 1500 ml :

0.4%	skimmed milk KS	6g		After autoclaving in the thorax the runny skimmed milk homogenises.
0.2%	yeast extract	3g		
1%	starch	15g		
0.1%	$CaCl_2$	1.5g	PH 7.4	Divide up into 30 x 50 ml portions in 250ml flasks + 1 ml XAD per flask each time.
0.1%	$MgSO_4$	1.5g		
50mM	hepes	17.85g		
8 mg/l	Fe EDTA	12mg		

Inoculate Soce 90 clone , Soce 950 Epo + Soce 660 Epo
25 ml of each culture are required !

Addition of malonic acid diamide (malonamide) + succinate

- Soce 660 Epo
  4 (nur Überstand übersimpft) sehr gut
  4 sehr gut
  4 sehr gut

- HPLC, Di 21.5.

D: [redacted]

- HPLC von verschiedenen Proben bzw. Kulturen
- neu ansetzen in Flüssigkultur + Platte!

Soce 1241 Scr.
1198 - 45/30 mehrere Platten
1199
471 Epo
523 Epo
613 Epo

- Kontrolle der Soce's auf Platte — Pilze?
- Platten von Protokoll 44 in den Kühlraum geräumt
- 20 µl in HPLC-Röhrchen gegeben von Soce 1198 - 45/30 für Hr. Steinmetz zur Analyse

PROTOKOLL 45 MUSS NOCH MIT SOCE 1198 - 45/30 DURCHGEFÜHRT WERDEN
PROTOKOLL 37 AUCH! PROTOKOLL 44 AUCH!

- Protokoll 37

1,5 l E-Med. kochen

E-Medium für 1500 ml

0,4 l.	Magermilch KS	6 g	nach dem Autoklavieren im Thorax die gesamte Magermilch homogenisieren
0,2 l.	Yeast extract	3 g	
1 l.	Stärke	15 g	
0,1 l.	$CaCl_2$	1,5 g	auf 30 250 ml Kolben à 50 ml aufteilen + jeweils 1 ml KAD/Kolben
0,1 l.	$MgSO_4$	1,5 g	
50 mM	Hepes	17,85 g	
8 mg/l	Fe-EDTA	12 mg	

pH 7,4!

Soce 90 klon, Soce 950 Epo + Soce 660 Epo animpfen.
Von jedes Kultur werden 25 ml benötigt!
Zugabe von Malonsäurediamid (Malonat) + Succinat (Bernsteinsäure)

Exhibit 3-5

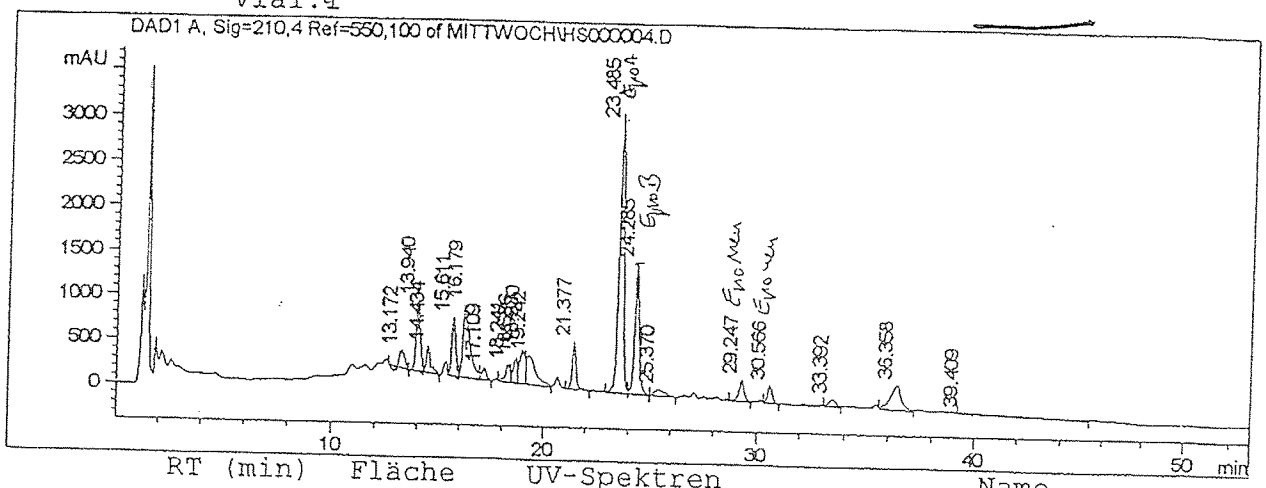
Epo new
Epo neu

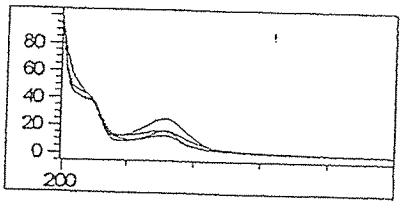
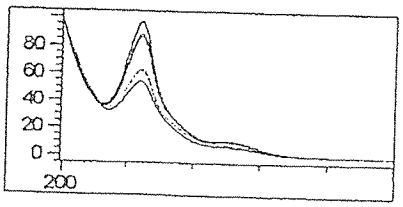
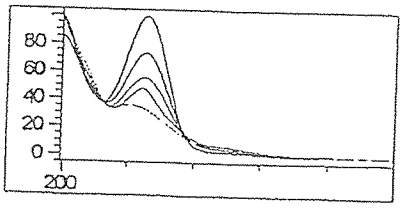
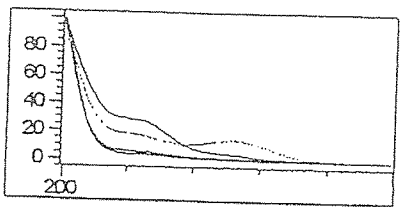
Epo new
Epo neu

Test Layout (Original-File: Spectra.rkf)

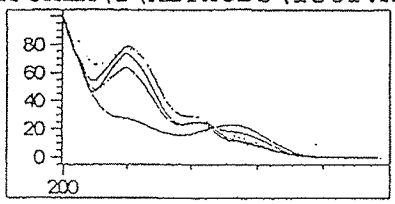
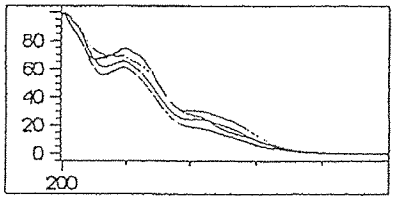
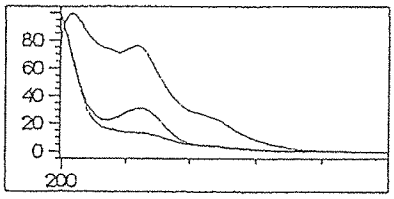
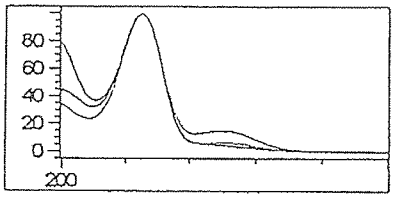
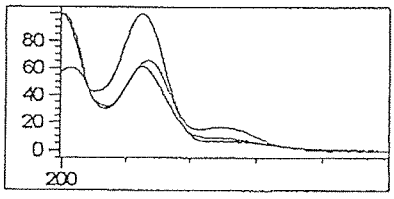
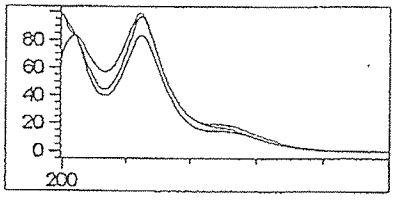
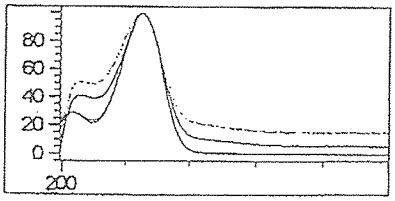
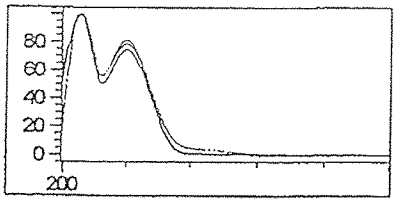
Data File name: C:\HPCHEM\1\DATA\MITTWOCH\HS00000->
Method name: C:\HPCHEM\1\METHODS\ISO1.M
Sample Name: So 1198/ 2. pos Sample Info: HPLC_MS_ ->
Injection Time: 10:39:32 AM
Sequence Name:
Report Style: screen1
data acquired by: Antje on: [redacted] 10:39:32 AM
vial: 9

5



RT (min)	Fläche mAU*sec	UV-Spektren	Name
13.17	3093.6		
13.94	11739.9		
14.43	4049.8		
15.61	9168.1		

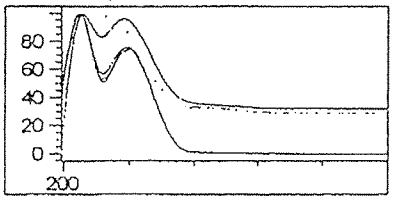
Data File name: C:\HPCHEM\1\DATA\M111WOCH\HS00000-2
Method name: C:\HPCHEM\1\METHODS\ISO1.M

16.18	19416.5	
17.11	2073.1	
18.24	3374.3	
18.59	3811.6	
18.93	7105.5	
19.24	10912.9	
21.38	7113.0	
23.48	47866.2	

Epoa

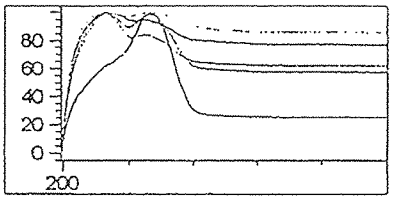
Data File name: C:\HPCHEM\1\DATA\MITTWOCH\HS00000->
Method name: C:\HPCHEM\1\METHODS\ISO1.M

24.29 21613.5

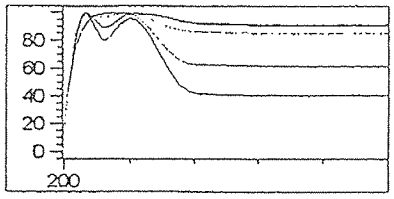


Eno3

25.37 2684.5

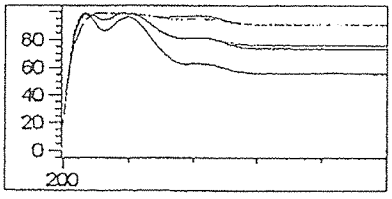


29.25 4218.0



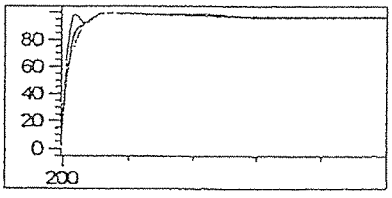
Eno neu
(M7= -16)

30.57 2971.2

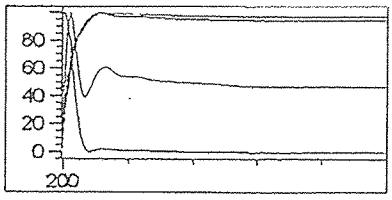


Eno neu
(M7= -16)

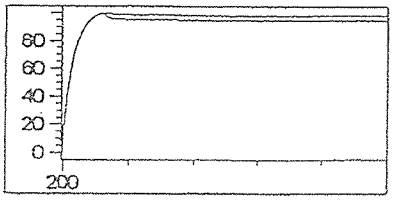
33.39 2040.5

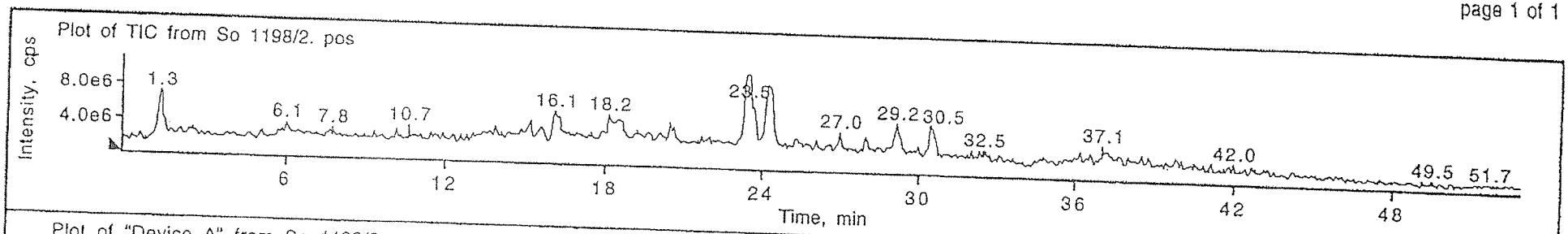


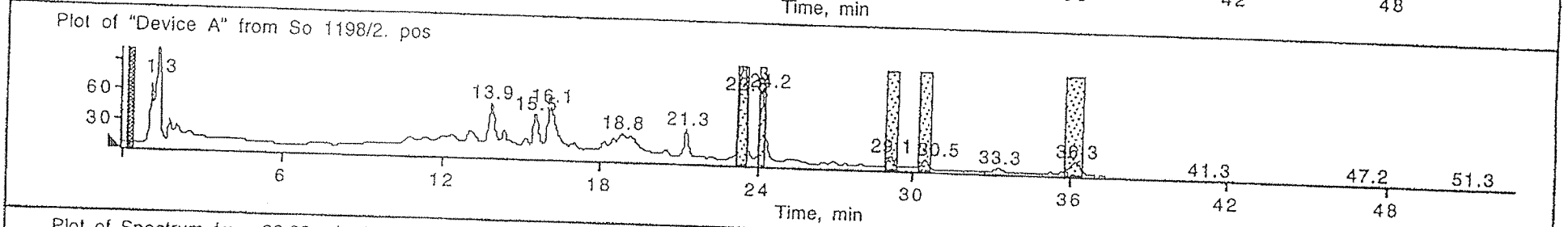
36.36 10485.2

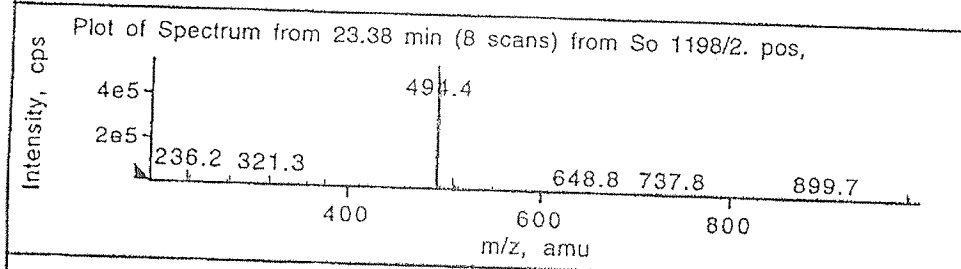


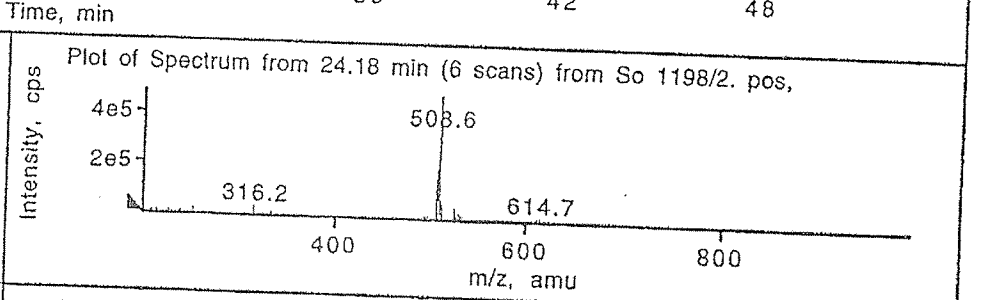
39.41 5935.8

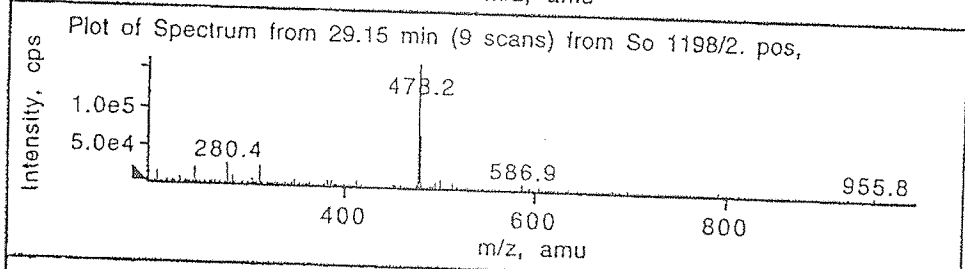


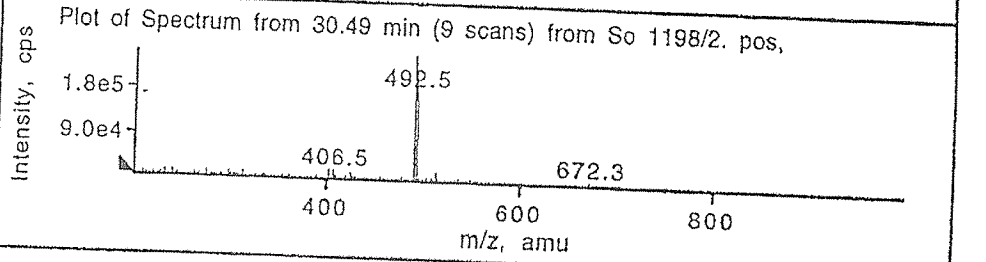

Plot of TIC from So 1198/2. pos
Intensity, cps
8.0e6
4.0e6
1.3
6.1
7.8
10.7
16.1
18.2
27.0
29.2
30.5
32.5
37.1
42.0
49.5
51.7
6
12
18
24
30
36
42
48
Time, min


Plot of "Device A" from So 1198/2. pos
60
30
1.3
13.9
15.5
16.1
18.8
21.3
33.3
41.3
47.2
51.3
6
12
18
24
30
36
42
48
Time, min


Plot of Spectrum from 23.38 min (8 scans) from So 1198/2. pos,
Intensity, cps
4e5
2e5
236.2
321.3
494.4
648.8
737.8
899.7
400
600
800
m/z, amu


Plot of Spectrum from 24.18 min (6 scans) from So 1198/2. pos,
Intensity, cps
4e5
2e5
316.2
508.6
614.7
400
600
800
m/z, amu


Plot of Spectrum from 29.15 min (9 scans) from So 1198/2. pos,
Intensity, cps
1.0e5
5.0e4
280.4
478.2
586.9
955.8
400
600
800
m/z, amu


Plot of Spectrum from 30.49 min (9 scans) from So 1198/2. pos,
Intensity, cps
1.8e5
9.0e4
406.5
492.5
672.3
400
600
800
m/z, amu

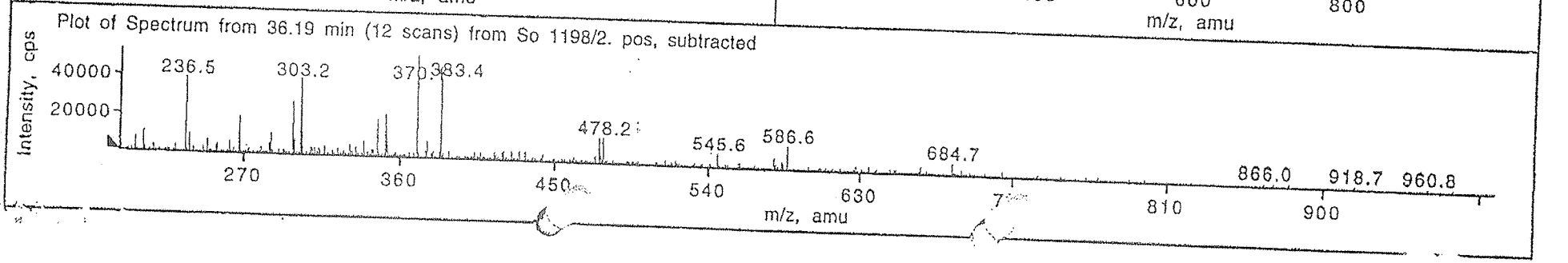

Plot of Spectrum from 36.19 min (12 scans) from So 1198/2. pos, subtracted
Intensity, cps
40000
20000
236.5
303.2
370.3
383.4
478.2
545.6
586.6
684.7
866.0
918.7
960.8
270
360
450
540
630
810
900
m/z, amu

Exhibit 3-6

6

EXHIBIT

DECLARATION OF TRANSLATOR

I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

do hereby avow and declare that I am conversant with the English and German languages and am a competent translator of German into English. I declare further that to the best of my knowledge and belief the following is a true and correct translation prepared and reviewed by me of the document in the German language attached hereto.

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Date: 5. August 2003

Confidential

Minutes no. 230 of meeting held at 09.00 on [redacted]

Present: Frau Kunze, Herr Augustiniak, Forche, Gerth, Höfle, Irschik, Jansen (not full time due to EDP Committee), Reichenbach, Sasse, Steinmetz, Washausen.

Epothilone (Reichenbach): During a visit to **Asta Medica**, first test results were presented. They showed that epothilone A and B had similar or better action than Taxol on four selected tumor cell lines. Epothilone B was up to ten times more potent. The LD_{50} in mice was determined to be ca. 100 mg/kg. In the case of a rapidly growing leukemia , a therapeutic dose of 20 - 30 mg/kg was able to achieve approximately 40% prolongation of life, a value similar to that of the standard cyclophosphamide. There is great interest in testing epothilone further and developing it as a cytostatic agent. Preclinical trials would require 10 - 20 grams of substance, and ca. 100 g - 1kg would be required up to clinical phase II. Authorisation could be applied for in three years in most favourable circumstances.

Testing of epothilones at **Boehringer Mannheim** has been delayed due to their tumor research being transferred to Italy. Nonetheless 100 mg of Epo A has been supplied, with which an in-vivo study in Mannheim will be run in parallel. **Bayer AG** has also expressed interest in epothilone and received 10 mg Epo A for initial trials. They are not primarily interested in using the natural product, but in derivatives for drug targeting. **Behringwerke** became aware of epothilone from a newspaper article and will be getting in touch with us shortly. Following a suggestion from Prof. Flohé, the US company **Sugen** has requested and already received 2 mg epothilone. **Bristol-Meyers-Squibb** has made an order for 100 mg epothilone. The state of testing at **Upjohn-Pharmacia** is not known. According to information from various sources, **Merck, Sharp and Dohme** stopped work on epothilone some time ago. **Ciba-Geigy** continues to express interest but has not yet made any definite orders.

Ciba test results (Reichenbach): Testing of Condramid is complete, the results do not require further work and the substance will shortly be released. The second attempt to carry out testing of Thuggacine against *Mykobakterium tuberculosis* in England again went wrong. Ciba will provide further material from its own supplies.

Epothilone (Steinmetz): Stocks of A/B mixture have decreased to 0.4 g after 100 mg were again used for derivatisation. The mixture needs further cleaning before using for test purposes. About 1-2 g epothilone mixture are available as raw extract.

Epothilone (Gerth): A 700 litre F-24 fermentor was run with So ce90 under sterile conditions but hardly produced anything. It was channelled using 0.5 mg/l epothilone and 6 - 7 mg Spirangien. The preculture for this fermentor had produced ca. 20 mg/l epothilone according to expectations, however. Another fermentor for producing epothilone using strain So ce660 is planned to run next week. This strain only produces Epo A and Spirangiens.
We have now been successful in plating strain So ce90. As already discovered for other strains, 10% of an old autoclaved culture has to be added to the strain. The usual procedures for optimising the strain can now be implemented.
It is very important that we should try to incorporate butyrate instead of acetate and propionate in the epoxide area, following the concept of mutasynthesis. The resulting ethyl analogue of epothilone could be more biologically active and would be patentable (ask Herr Bqeters).

The following strains have been identified as new epothilone producers: So ce611, So ce498, So ce931, So ce618, So ce414, So ce320 and So ce1087. All these strains are less effective producers and also form Spirangins or Icumazole. An exception is strain So ce1198, which in addition to epothilones A and B also produces a small quantity of an unknown substance with two peaks in the uV spectrum that are more lipophilic than the epothilones. According to HPLC/MS studies carried out by Herr Steinmetz, the substance is composed of homologues (ΔM 14*) possessing one oxygen less than epothilone A and B. There were ca. 1-2 mg of the new epothilones present in the shaken culture. They should be isolated individually or as a mixture. According to the NMR measurement, a biological test should be carried out.
While optimising the media, the strains So ce90, So ce660 and So ce950 were cultivated in 10 different media. There were significant variations in growth and production between different strains.

* (handwritten note) ΔM 14 is typing mistake, should be ΔM 16

6

Vertraulich

Protokoll Nr. 230 der Besprechung vom [redacted], 9.00 Uhr

Teilnehmer: Frau Kunze, die Herren Augustiniak, Forche, Gerth, Höfle, Irschik, Jansen (zeitweise wegen EDV-Kommission), Reichenbach, Sasse, Steinmetz, Washausen.

Epothilon (Reichenbach): Bei einem Besuch bei der **Asta Medica** wurden erste Versuchsergebnisse vorgestellt. Danach wirken Epothilon A und B bei vier ausgewählten Tumorzellinien ähnlich oder besser als Taxol. Epothilon B erwies sich dabei bis zu 10x aktiver. Die LD_{50} in der Maus wurde zu ca. 100 mg/kg bestimmt. Im Fall einer schnell wachsenden Leukämie konnte mit einer therapeutischen Dosis von 20 - 30 mg/kg eine ca. 40%ige Lebensverlängerung erzielt werden, ein Wert, der dem Standard Cyclophosphamid entspricht. Es besteht großes Interesse, Epothilon exclusiv weiter zu testen und als Cytostatikum zu entwickeln. Für Vorklinische-Versuche würden 10 - 20 Gramm Substanz, bis zur klinischen Phase II, ca. 100 g - 1kg benötigt. Eine Zulassung könnte im günstigsten Fall in drei Jahren beantragt werden.

Bei **Boehringer** Mannheim hat sich die Testung der Epothilone verzögert, da die Tumorforschung nach Italien verlegt worden ist. Es wurden jedoch 100 mg Nachsubstanz Epo A geliefert, mit denen parallel eine in-vivo Studie in Mannheim durchgeführt wird. Die **Bayer AG** hat ebenfalls Interesse an Epothilon bekundet und für erste Versuche 10 mg Epo A erhalten. Dort ist man nicht an der Anwendung des Naturstoffs primär interessiert, sondern an Derivaten im Sinne von Drug-Targeting. Die **Behringwerke** sind durch einen Zeitungsartikel auf Epothilon aufmerksam geworden und werden demnächst mit uns Kontakt aufnehmen. Auf einen Hinweis von Prof. Flohé hat die Firma **Sugen** (USA) um 2 mg Epothilon A gebeten und es bereits bekommen. Von **Bristol-Meyers-Squibb** liegt eine Bestellung für 100 mg Epothilon vor. Der Stand der Testung bei **Upjohn-Pharmacia** ist nicht bekannt. Nach Informationen aus verschiedenen Quellen hat **Merck, Sharp and Dohme** die Bearbeitung von Epothilon bereits seit längerer Zeit aufgegeben. **Ciba-Geigy** ist weiterhin interessiert, allerdings ist bis jetzt keine konkrete Substanzbestellung eingegangen.

Testergebnisse Ciba (Reichenbach): Die Testung von Condramid ist abgeschlossen, die Ergebnisse rechtfertigen keine weitere Bearbeitung, und die Substanz wird demnächst frei-

gegeben. Auch beim zweiten Anlauf ist die Testung des Thuggacins gegen *Mykobakterium tuberculosis* in England schiefgegangen. Die Ciba wird Nachsubstanz aus dem eigenen Vorrat bereitstellen.

Epothilon (Steinmetz): Der Vorrat an einem A/B-Gemisch ist auf 0,4 g geschrumpft nachdem für die Derivatisierung wieder 100 mg verbraucht worden sind. Für eine Abgabe zu Testzwecken muß das Gemisch noch weiter gereinigt werden. Als Rohextrakt liegen ca. 1-2 g Epothilongemisch vor.

Epothilon (Gerth): Ein 700 ℓ Fermenter F-24 mit Soce 90 ist steril gelaufen, hat jedoch kaum produziert. Er wurde bei 0.5 mg/ ℓ Epothilon und 6 - 7 mg Spirangien kanalisiert. Die Vorkultur für diesen Fermenter hatte allerdings mit ca. 20 mg/ ℓ Epothilon erwartungsgemäß produziert. Nächste Woche soll ein weiterer Fermenter zur Herstellung von Epothilon mit Stamm Soce 660 laufen. Dieser Stamm produziert nur Epo A und Spirangiene.
Es ist jetzt gelungen den Stamm Soce 90 zu plattieren. Dazu muß, wie bereits früher bei anderen Stämmen gefunden, 10% einer alten, autoklavierten Kultur des Stammes zugegeben werden. Damit können nun die üblichen Verfahren zur Stammoptimierung eingesetzt werden.
Sehr wichtig ist es, u.a. zu versuchen, im Sinne einer Mutasynthese statt Acetat und Propionat im Bereich des Epoxids Butyrat einzubauen. Das resultierende Ethylanaloge Epothilon könnte biologisch aktiver sein und wäre patentierbar (bei Herrn Boeters nachfragen).

Als neue Epothilonproduzenten wurden identifiziert: Soce 611, Soce 498, Soce 931, Soce 618, Soce 414, Soce 320 und Soce 1087. Alle diese Stämme sind schlechtere Produzenten und bilden daneben Spriangiene oder Icumazole. Eine Ausnahme bildet der Stamm Soce 1198 der neben Epothilon A und B eine unbekannte Substanz und in geringer Menge zwei lipophilere Peaks mit dem UV-Spektrum der Epothilone. Nach HPLC/MS -Untersuchungen von Herrn Steinmetz handelt es sich dabei um Homologe (ΔM 14) die einen Sauerstoff weniger als Epothilon A und B besitzen. In der vorliegenden Schüttelkultur liegen ca. 1-2 mg der neuen Epothilone vor. Sie sollen einzeln oder als Gemisch isoliert werden. Nach der NMR-Messung soll ein biologischer Test versucht werden.
Bei einer Medienoptimierung wurden die Stämme Soce 90, Soce 660 und Soce 950 in 10 verschiedenen Medien kultiviert. Die Variation von Wachstum und Produktion waren groß und bei den einzelnen Stämmen unterschiedlich.

ΔM14 ist Schreibfehler muß ΔM16 sein

Exhibit 3-7

7

EXHIBIT

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Date: 5. August 2003

[redacted]

Soce 1198 – 45/30 / Screening / [redacted]

7

MeOH – extract : Weight: 198g mg

(Illegible) (Illegible)

(HPLC Test Result) (HPLC Test Result)

95 CH_2Cl_2 / 5 MeOH 95 CH_2Cl_2 / 5 MeOH

[redacted]

→ LH-20 – Separation

Column = LH-20, ≅70 cm long, diam. 1.5 cm

Solvent = MeOH, λ = 227 nm

Flow = 1.4 ml/ min , Range = 0.1 –

Paper = 2mm / min , Fractionation time = 3 min

Fractionation

~~LH—1~~ = gl. 1-9 = — HPLC

~~LH—2~~ = gl. 10-17 = Weight 82mg → RP separation [redacted] HPLC

~~LH—3~~ = gl. 18-23 = — HPLC

~~LH—4~~ = gl. 24-30 = — discarded HPLC

~~LH—5~~ = gl. 31-41 = — HPLC

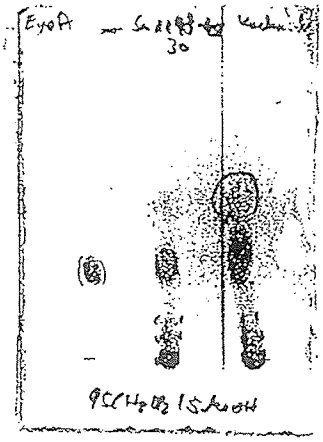
~~LH—6~~ = gl. 42-51 = — HPLC

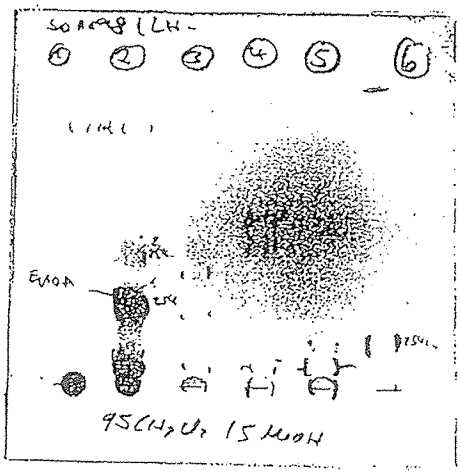
Socc 1198-45/30 /Screening [redacted]

Sorangium cellulosum
Socc 1198-45/30
Screening!
K. Gerth: 02.05.96.

MeOH-Extrakt: Gewicht: 198 mg

7





[redacted]

→ LH-20-Trennung

Säule = LH-20, ≈ 70 cm lang, Ø 1,5 cm

LM = MeOH, λ = 227 nm

Fluß = 1,4 ml/min, Range = 0.1-

Papier = 2 mm/min, Fraktionierzeit = 3 min

Fraktionierung

~~LH-1~~ = Fr. 1-9 = HPLC

~~LH-2~~ = Fr. 10-17 = HPLC Gewicht = 82 mg → RP-Trennung [redacted]

~~LH-3~~ = Fr. 18-23 = HPLC

~~LH-4~~ = Fr. 24-30 = HPLC

~~LH-5~~ = Fr. 31-41 = HPLC

~~LH-6~~ = Fr. 42-51 = HPLC

verworfen

Exhibit 3-8

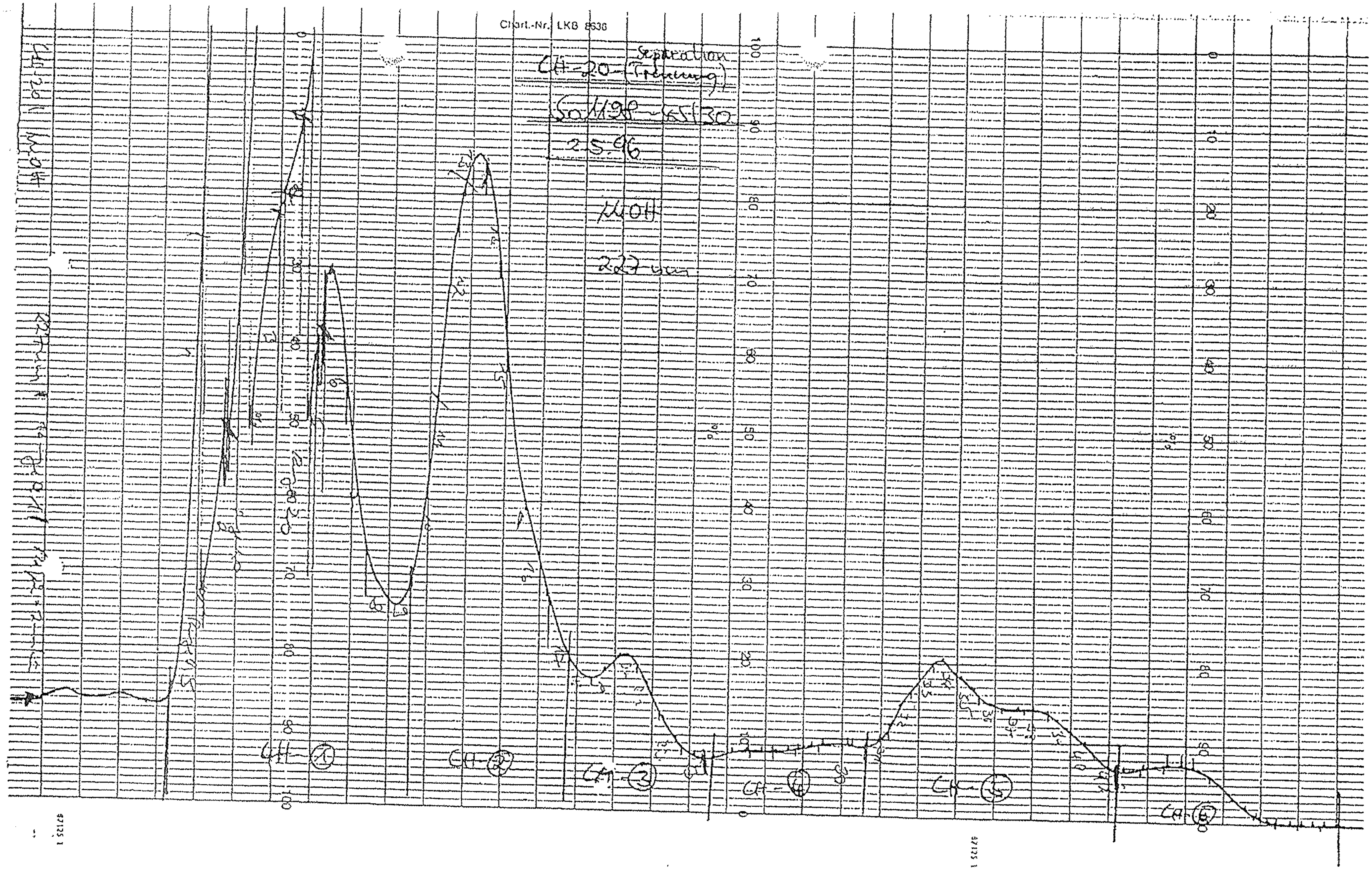


Chart-Nr. LKB 8536
CH-20-(Trennung) separation
2.5.96
MeOH
227 nm
CH-1
CH-2
CH-3
CH-4
CH-5
CH-6
87125 1
87125 1

8

Exhibit 3-9

Sample: So1198_LH_2

Report Method: __Spectrum__Index_Plot Printed: [redacted]

Project Name:	Silke_1	Sample Type:	Unknown
Sample Name:	So1198_LH_2	Vial:	3 Inj. 1
Date Acquired:	12.06.96 10:57:38	Volume:	3.00
Date Processed:	12.06.96 11:59:31	Run Time:	20.0 min
SampleWeight:	1.00000	Laufmittel:	76MeOH/24H2O,NH4Ac
Dilution:	1.00000		
Channel:	996 PDA 210.0 nm		
Acq Meth Set:	Sora_MS_210nm		
Processing Method:	Epothilon_210_PM		

9

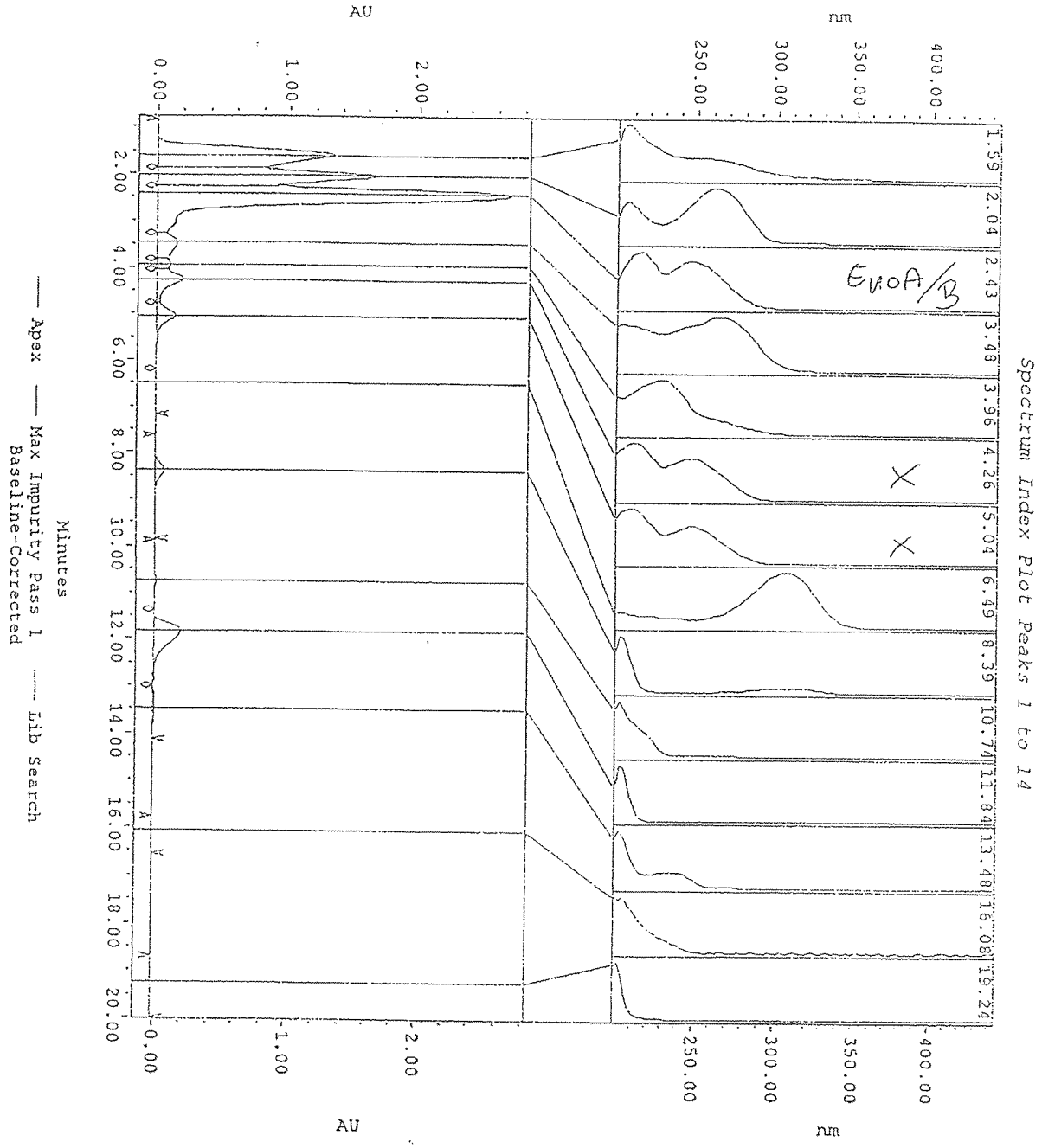


Exhibit 3-10

10

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R Richards

Date: 5. August 2003

RP separation of So1198 – LH-2

So1198 LH-2 Weight: 82mg separated in 2 runs

Column = Nucleosil 100 C18, 7µm , 20 x 250 mm

Solvent = 73 MeOH / 27 H_2O +0.05M NH_4Ac

λ = 210 nm , Range = 016 - 128

Pump = 200 , Paper = 5 mm/ min

Fractions concentrated up to H_2O phase, extracted 2 x with EE, EE phase washed with H_2O and dried with Na_2SO_4.

(Test Result)

95 CH_2Cl_2 / 5 MeOH Sprayed with vanillin – H_2SO_4

Epo A/B = 2mg/ml

Fractionation

~~So 1198 – RP 1~~ = Weight 0.7 mg , NMR 002549 , .. 660 ..
→ 0.1 mg 2nd test 150 ng/ ml

→ prep. DC 19.6.96

So 1198 – RP –2 = Weight: 1.0 mg, NMR 002550, .. 661 ..
COSY
→ 0.1mg 2nd test 100 ng/ml

10

RP-Trennung von So1198-CH-②

So1198-CH-② = Gewicht: 82mg in 2 Läufen getrennt

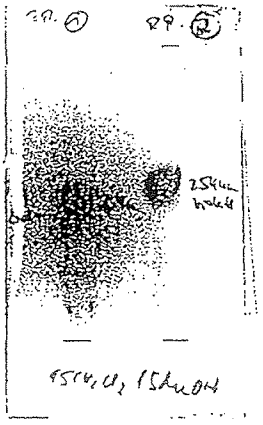
Säule = Whatman 100 C18, 7µm, 20×250mm

LM = 73 MeOH 127 H_2O 1+0,05M NH_4Ac

λ = 210nm, Range: 0,16 - 1,28

Pumpe = 200, Papier = 5mm/min

Fraktionen bis zum H_2O-Peak eingengt, 2× mit EE extrahiert, EE-Phase mit H_2O gewaschen und mit Na_2SO_4 getrocknet.



a. gesprüht mit Vanillin-H_2SO_4

Epo A B = 2mg/ml

Fraktionierung:

So1198-RP-① = Gewicht: 0,7mg, NMR 00 2549, Ubr. 660qu
→ 0,1mg z. Test 150ng/ml

→ präp. DC (19.6.96)

So1198-RP-② = " : 1,0mg, NMR 00 2550, Ubr. 661qu
cosy
→ 0,1mg z. Test 100ng/ml

Exhibit 3-11

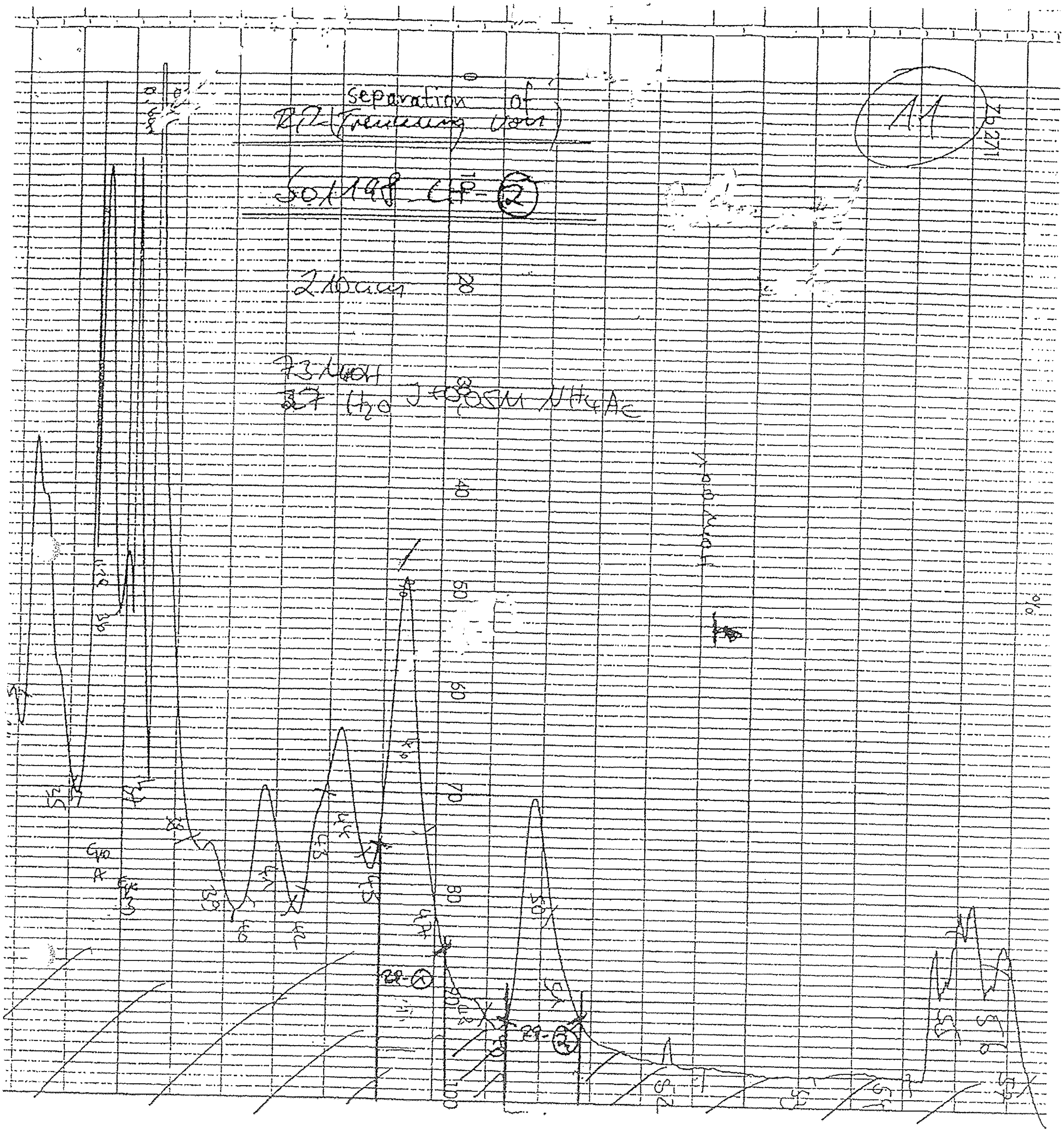

Separation of
(Trennung von)
50 1198 CP-2
210 nm
73 MeOH
27 H2O] 0.05M NH4Ac
11

Exhibit 3-12

EXHIBIT

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Ronald Richards

Date: 5. August 2003

11

12

NMR REQUEST

GBF – Dept. of Molecular structure research

Date received: [redacted]
Spectrum no. 002550

Substance name: So 1198 – RP-2
Substance producer: Pohlaus
Dept.: NC (1.1-2) tel. 343
Nuclear species: $^1H_1$
Amount of substance : 1.0 mg
Suitable solvent: CD_3OD
Return substance? Yes

General Information
Store sample in fridge Y

Signal expected between
$\delta = 0$ and 9
Requested: only spectra Y
plus integral Y

Type of experiment

$^1H_1$ Standard spectrum Y

Plot and Data manipulation

$\delta = 8.9$ to — 0.1 (0.15 ppm/cm) Y

Special requests: COSY Y

Measured on AM-300 Y
Filed under no. SIPZ 2550110/ + COSY

Einlieferungsdatum: [redacted]

Spektren-Nr.: 002550

[redacted]

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

Substanz-Bez.: Sa1198-77-2

Summenformel: _____

Substanzhersteller: Roche

Abteilung: NC (A.1-7) Tel.: 343

Kernart (¹H), ¹³C, ³¹P, andere?) _____

Substanz-Menge: 1,0 mg, Molmasse: _____

geeignetes Lösungsmittel: CD3OD weitere Messung nach Zugabe von _____

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒

im Tiefkühlfach ☐

im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen

δ = 0 und 9

Gewünscht: nur Spektrum ☒

plus Integral ☒

Interpretation ☐

Zahl der Akkumulationen (falls > 104): _____

Art des Experiments

[¹H] Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en): _____

[¹³C] ¹H-Entkopplung:

Breitband ☐ selektiv ☐

DEPT ☐ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

Linienausdruck ☐

[¹H]

δ = 8.9 bis —0.1 (0.15 ppm/cm) ☒

11.9 bis —0.1 (0.2 ppm/cm) ☐

Drehungen:

10 Hz/cm ☐ von δ = _____ bis _____

[¹³C] normal (δ = 220 bis 0) ☐

anderes Format: _____

Sonderwünsche: COSY ☒

¹³C — ¹H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☒ AM-300 ☐ ARX-400 ☐ DMX-600

gespeichert unter Nr. SPE250140 / m cay

Bitte um Rücksprache ☐

Kommentar:

(Unterschrift)

Sulke reports; NMR runs
Spe brings to Haefle for interp.
He interprets

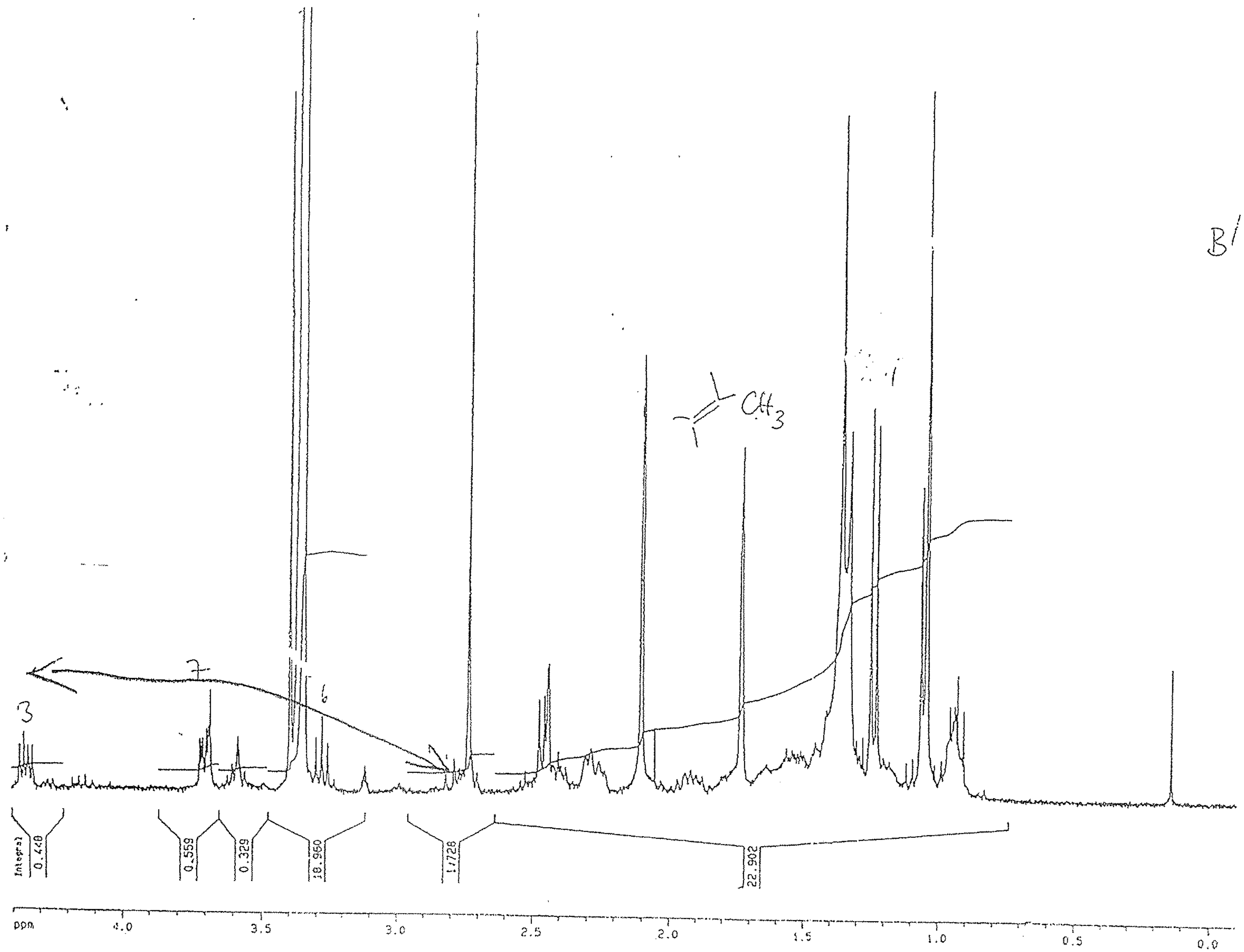
So 1198

RP- ②

1mg

⑫

SIPZ2550 10 1 Pohlan



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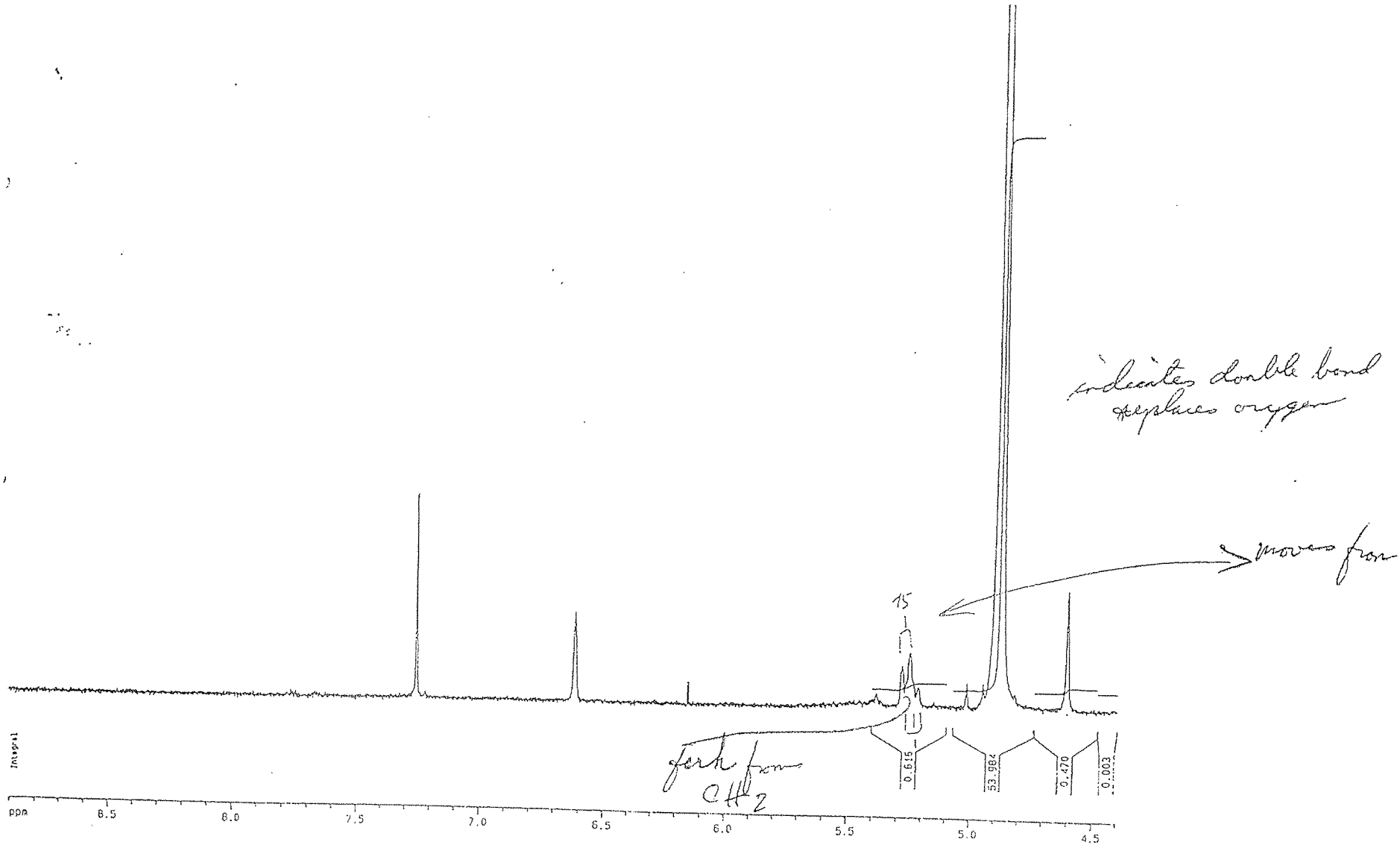
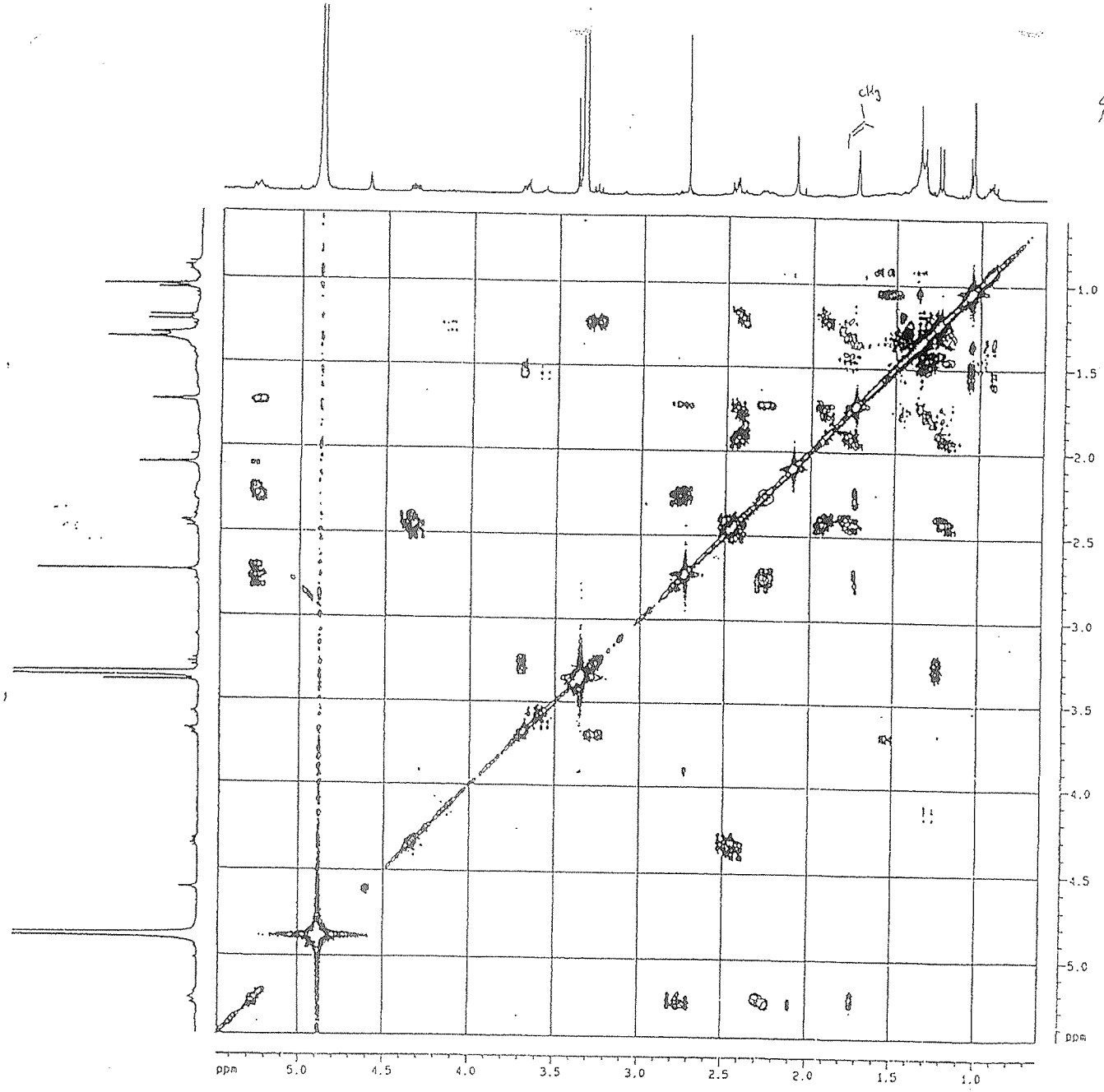
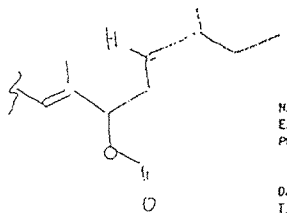
PROCNO	1
F2 - Acquisition Parameters	
Date_	[redacted]
Time	4.55
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	246
DS	0
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	812.7
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	14.50 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-3.00 dB
F2 - Processing parameters	
SI	16384
SF	300.1300074 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00
1D NMR plot parameters	
CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm

SIPZ2550 10 1 Pohlan



27-(2)





Current Data Parameters

NAME	SIPZ2550
EXPNO	11
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	7.47
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	cosy45gs
TD	2048
SOLVENT	MeOH
NS	72
DS	16
SWH	2495.910 Hz
FIDRES	1.216267 Hz
AQ	0.4104692 sec
RG	9195.2
DW	200.400 usec
DE	4.75 usec
TE	303.0 K
d0	0.0000030 sec
d13	0.0000030 sec
D1	1.00000000 sec
P1	14.50 usec
P16	1000.00 usec
D16	0.00020000 sec
DE	4.75 usec
SF01	300.1311613 MHz
NUC1	1H
PL1	-3.00 dB
INO	0.000400B0 sec

F1 - Acquisition parameters

NDO	1
TD	512
SF01	300.1312 MHz
FIDRES	4.873087 Hz
SW	8.313 ppm

F2 - Processing parameters

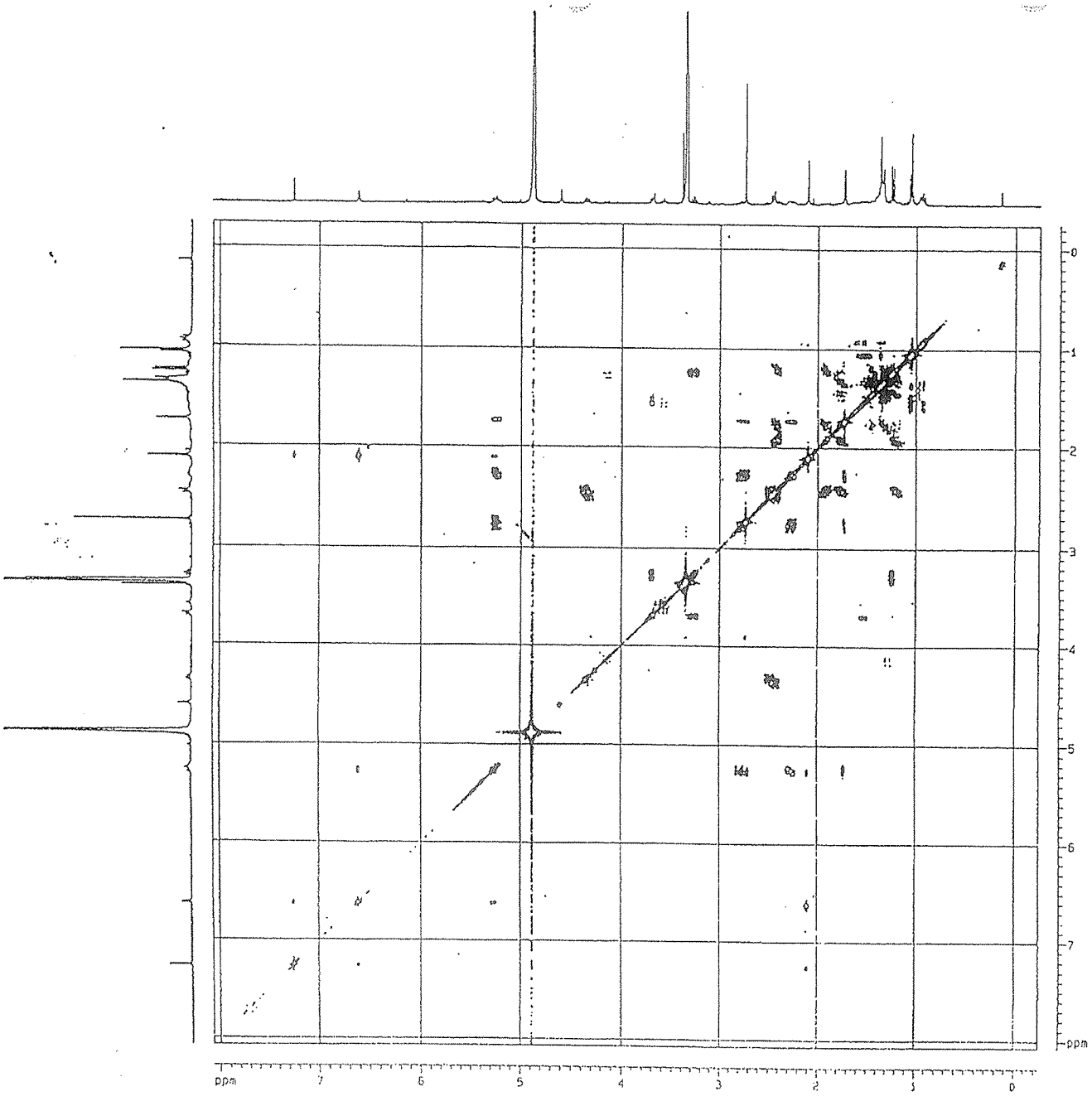
SI	2048
SF	300.1300074 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

F1 - Processing parameters

SI	1024
MC2	QF
SF	300.1300074 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	20.00 cm
CX1	20.00 cm
F2PLO	5.470 ppm
F2LO	1641.66 Hz
F2PHI	0.623 ppm
F2HI	187.05 Hz
F1PLO	5.470 ppm
F1LO	1641.67 Hz
F1PHI	0.607 ppm
F1HI	182.18 Hz
F2PPMCM	0.24233 ppm/cm
F2HZCM	72.73051 Hz/cm
F1PPMCM	0.24314 ppm/cm
F1HZCM	72.97448 Hz/cm



Current Data Parameters

NAME	SIP22550
EXPNO	11
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	7.47
INSTRUM	spect
PROBHD	5 mm ONP 1H
PULPROG	cosy45ps
TD	2048
SOLVENT	MeOH
NS	72
DS	16
SWH	2495.010 Hz
FIDRES	1.218267 Hz
AQ	0.4104692 sec
RG	9195.2
DW	200.400 usec
DE	4.75 usec
TE	303.0 K
d0	0.0000030 sec
d13	0.0000030 sec
D1	1.00000000 sec
P1	14.50 usec
P16	1000.00 usec
D16	0.00020000 sec
DE	4.75 usec
SFO1	300.1311813 MHz
NUC1	1H
PL1	-3.00 dB
IN0	0.00040080 sec

F1 - Acquisition parameters

NDO	1
TD	512
SFO1	300.1312 MHz
FIDRES	4.873087 Hz
SW	8.313 ppm

F2 - Processing parameters

SI	2048
SF	300.1300074 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

F1 - Processing parameters

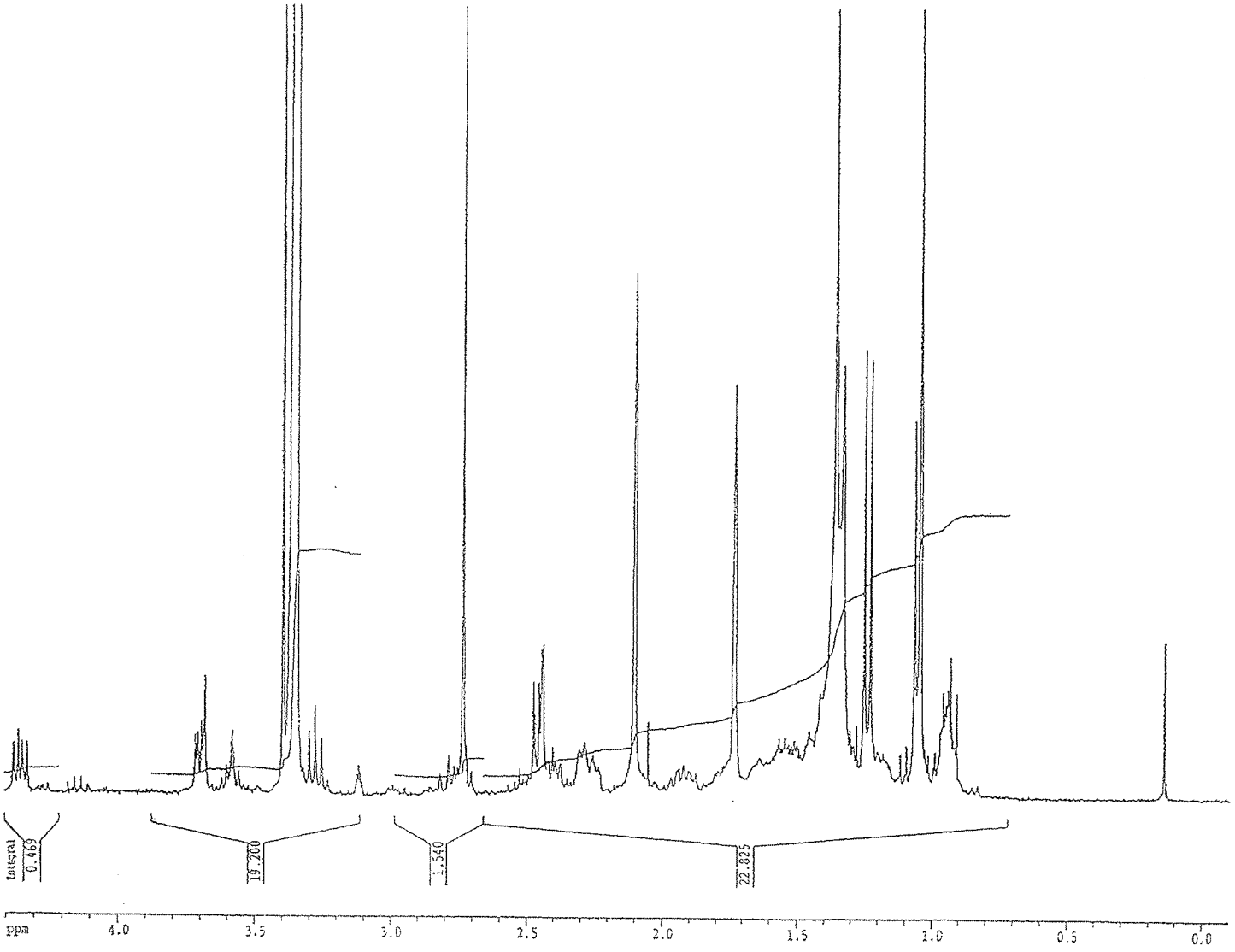
SI	1024
MC2	QF
SF	300.1300074 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	20.00 cm
CX1	20.00 cm
F2PLO	8.058 ppm
F2LO	2421.35 Hz
F2PHI	-0.245 ppm
F2HI	-73.66 Hz
F1PLO	8.058 ppm
F1LO	2421.36 Hz
F1PHI	-0.245 ppm
F1HI	-73.66 Hz
F2PPMCM	0.41505 ppm/cm
F2HZCM	124.75050 Hz/cm
F1PPMCM	0.41566 ppm/cm
F1HZCM	124.75102 Hz/cm

Epo D
NMR Pohlan sp.

SIPZ2550 10 1 Pohlan



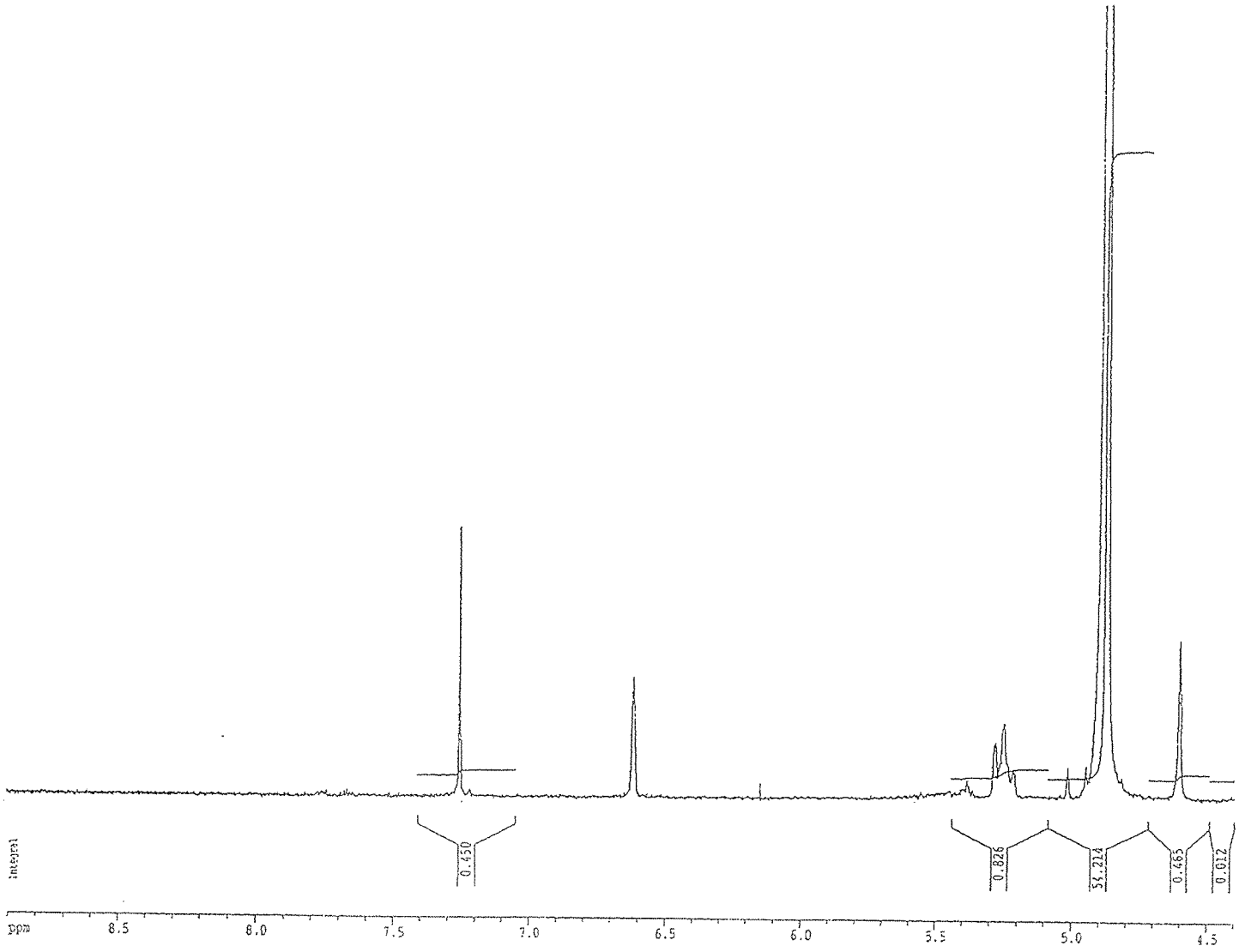
Current Data Parameters
NAME SIPZ2550
EXPNO 10
PROCNO 1

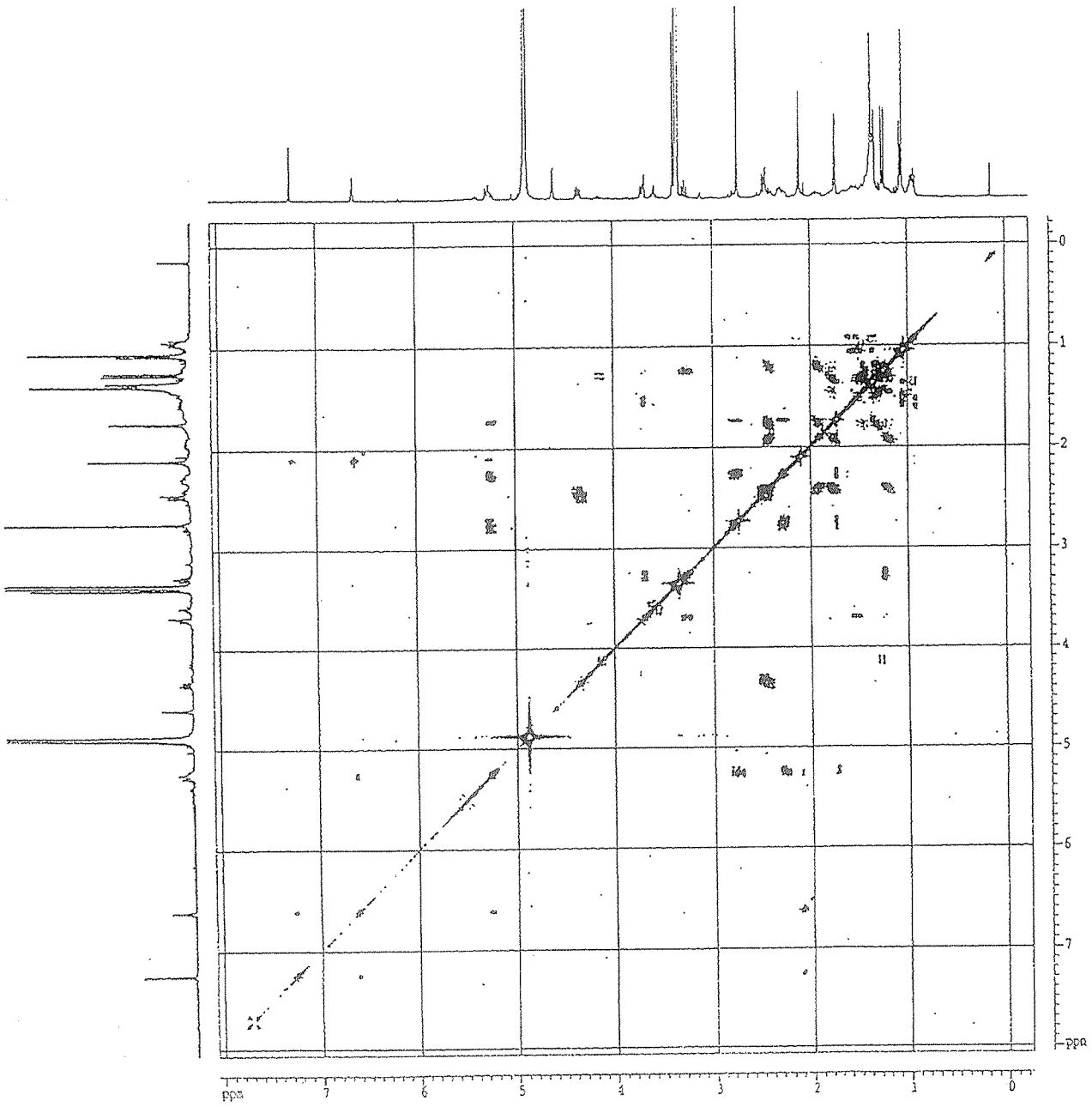
F2 - Acquisition Parameters
Date_ [redacted]
Time 13.55
INSTRUM spect
PROBHD 5 mm QNP 1H
PULPROG zg30
TD 32768
SOLVENT MeOH
NS 246
DS 0
SWH 6172.839 Hz
FIDRES 0.188380 Hz
AQ 2.6542580 sec
RG 812.7
DW 81.000 usec
DE 4.50 usec
TE 300.0 K
D1 1.00000000 sec
P1 14.50 usec
DE 4.50 usec
SFO1 300.1318534 MHz
NUC1 1H
PL1 -3.00 dB

F2 - Processing parameters
SI 32768
SF 300.1300076 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 30.00 cm
F1P 4.400 ppm
F1 1320.57 Hz
F2P -0.100 ppm
F2 -30.01 Hz
PPMCM 0.15000 ppm/cm
HZCM 45.01950 Hz/cm

SIPZ2550 10 1 Pohlan





Current Data Parameters

NAME	SIM2550
EXPNO	11
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	16.47
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	cosy45gs
TD	2048
SOLVENT	MeOH
NS	72
DS	16
SWH	2495.010 Hz
FIDRES	1.218267 Hz
AQ	0.4104692 sec
RG	9195.2
DW	200.400 usec
DE	4.75 usec
TE	303.0 K
d0	0.0000030 sec
d13	0.0000030 sec
D1	1.00000000 sec
P1	14.50 usec
P16	1000.00 usec
D16	0.00020000 sec
DE	4.75 usec
SFO1	300.1311813 MHz
NUC1	1H
PL1	-3.00 dB
IND	0.00040080 sec

F1 - Acquisition parameters

NDO	1
TD	512
SFO1	300.1312 MHz
FIDRES	4.873087 Hz
SW	8.313 ppm

F2 - Processing parameters

SI	2048
SF	300.1300076 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

F1 - Processing parameters

SI	1024
MC2	QF
SF	300.1300076 MHz
WDW	SINE
SSB	0
LB	0.00 Hz
GB	0

2D NMR plot parameters

CX2	20.00 cm
CX1	20.00 cm
F2PLO	8.067 ppm
F2LO	2421.16 Hz
F2PHI	-0.246 ppm
F2HI	-73.85 Hz
F1PLO	8.067 ppm
F1LO	2421.17 Hz
F1PHI	-0.246 ppm
F1HI	-73.85 Hz
F2PPMCM	0.41565 ppm/cm
F2HZCM	124.75050 Hz/cm
F1PPMCM	0.41566 ppm/cm
F1HZCM	124.75102 Hz/cm

Exhibit 3-13

13

EXHIBIT

DECLARATION OF TRANSLATOR

I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

do hereby avow and declare that I am conversant with the English and German languages and am a competent translator of German into English. I declare further that to the best of my knowledge and belief the following is a true and correct translation prepared and reviewed by me of the document in the German language attached hereto.

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Date: 5. August 2003

[redacted]

Preparative DC of So1198 / RP–1

So1198/ RP–1 = Weight = 0.6 mg put in CH_2Cl_2

KG_{60} F_{254nm}, diam. 0.2 mm , Al foil, 7 x 7 cm

DC solvent = 95 CH_2Cl_2 / 5 MeOH

front

Bands cut out, extracted 3 x with MeOH in centrifuge tube, oil pump, absorbed in CH_2Cl_2, filtered through cotton wool, concentrated, oil pump

(Test result)

(254nm)

So1198–RP-1 / DC Weight 0.4 mg, NMR 002630
→ 0.1 mg 2nd test

(Test result)

Sprayed with
vanillin – H_2SO_4

[redacted]

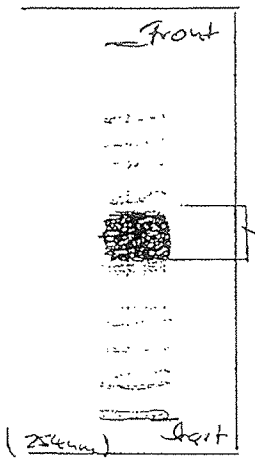
13

Präparatives DC von So1198 RP-①

So1198 RP-① = Gewicht: 0,6 mg in CH_2Cl_2 aufgetragen

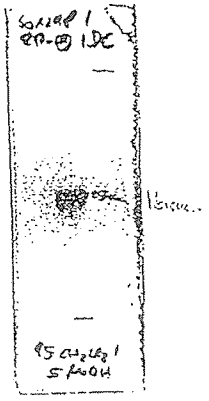
KG60 F254nm, Ø 0,2mm, Alufolie, 7 × 7cm

DC-LM: 95 CH_2Cl_2 15 MeOH



Bande herausgeschnitten, mit MeOH im Zentrifugenglas 3× extrahiert, MeOH-Extrakt eingeengt, Ölpumpe, in CH_2Cl_2 aufgenommen, über Watte filtriert, eingeengt, Ölpumpe.

So1198-RP-①/DC: Gewicht: 0,4 mg, NMR002630
→ 0,1 mg 2-Test



angesprüht mit Vanillin-H2SO4

Exhibit 3-14

EXHIBIT

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I, Ronald Richards, of the Technical Translation Agency, 2136 Laa/Thaya, Austria,

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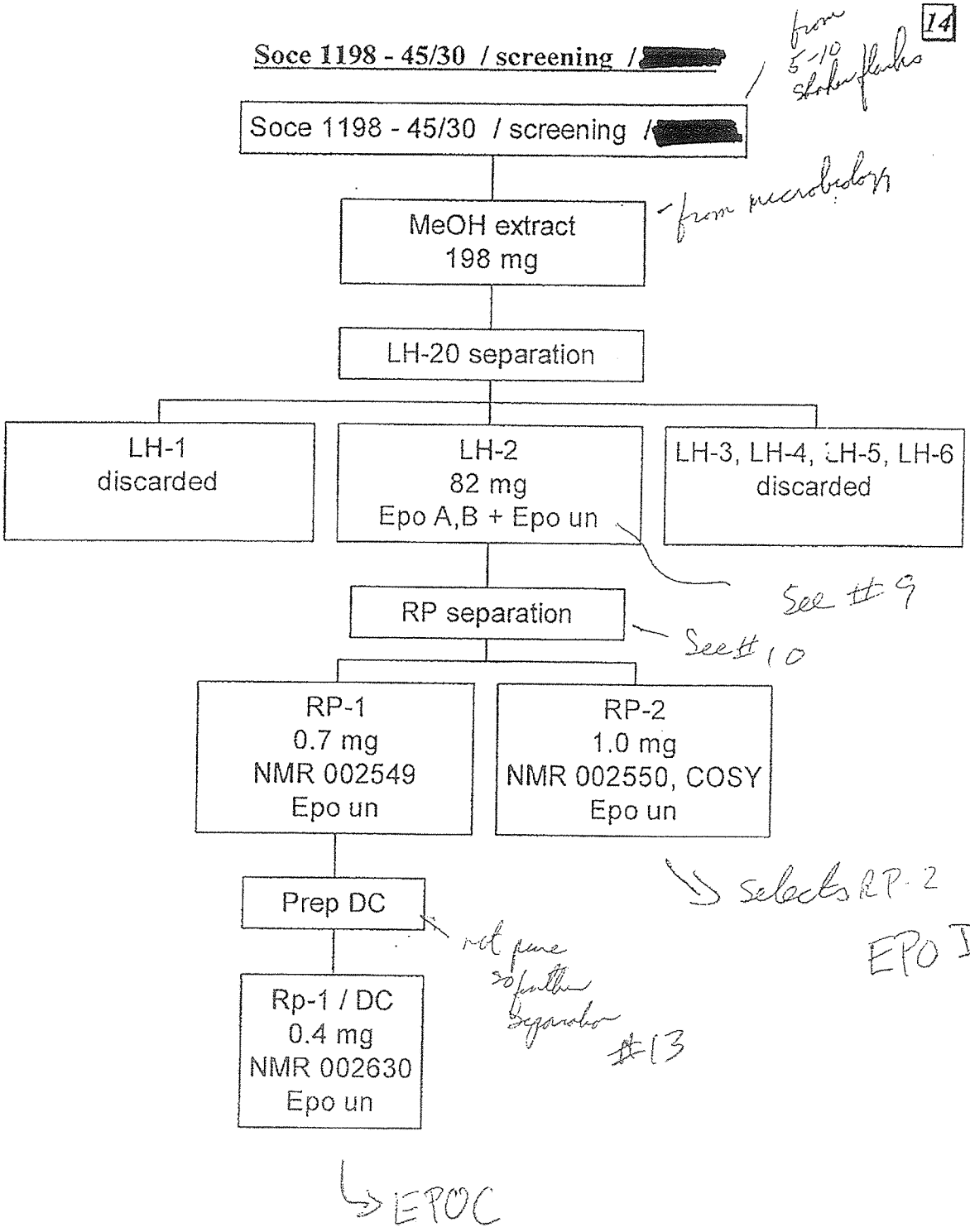
Date: 5. August 2003


Soce 1198 - 45/30 / screening / [redacted]
from 5-10 shaker flasks
Soce 1198 - 45/30 / screening / [redacted]
MeOH extract
198 mg
from microbioton
LH-20 separation
LH-1
discarded
LH-2
82 mg
Epo A,B + Epo un
LH-3, LH-4, LH-5, LH-6
discarded
See #9
RP separation
See #10
RP-1
0.7 mg
NMR 002549
Epo un
RP-2
1.0 mg
NMR 002550, COSY
Epo un
selects RP-2
EPO D
Prep DC
not pure
so further
separation
#13
Rp-1 / DC
0.4 mg
NMR 002630
Epo un
EPO C

(14)

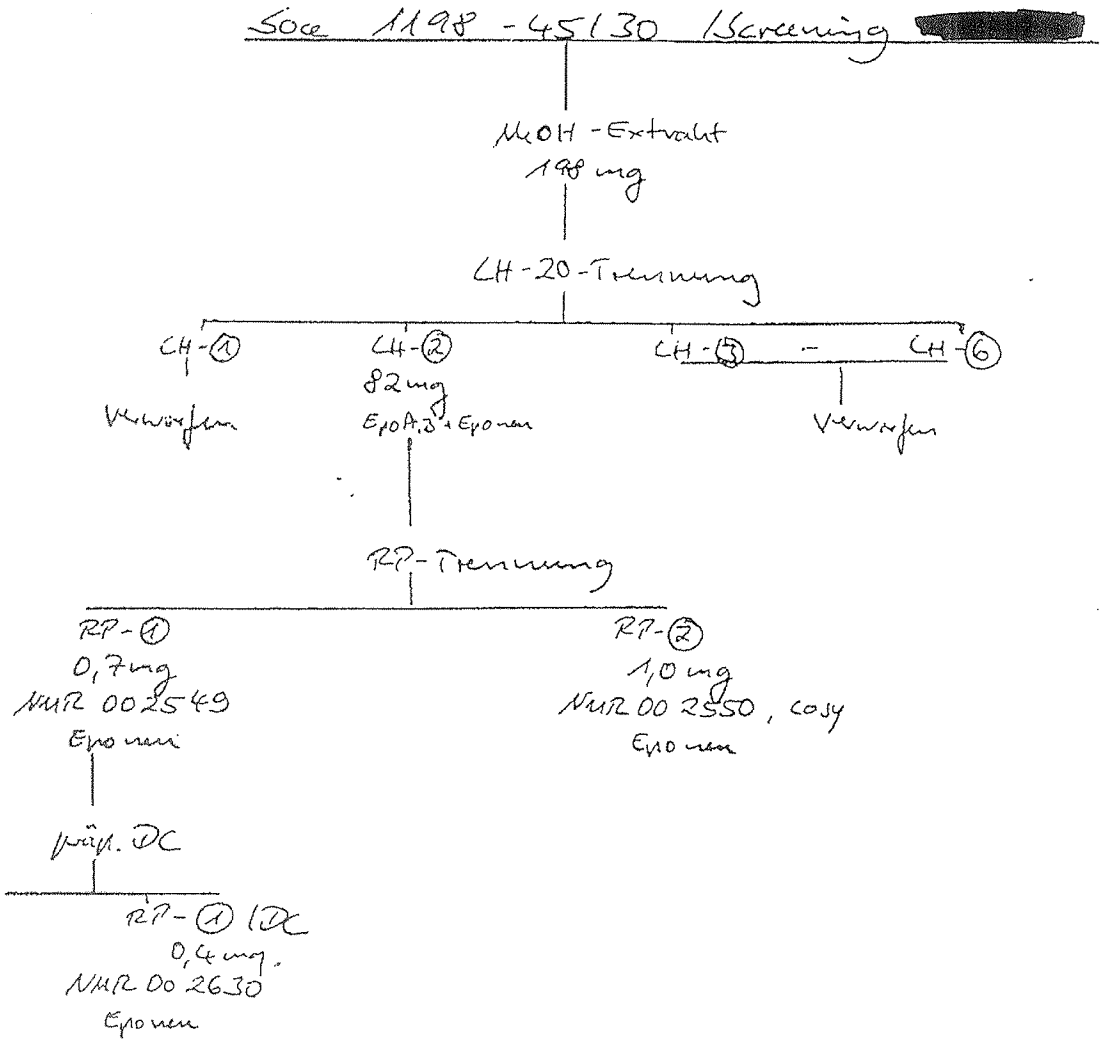

Soce 1198 - 45130 Screening [redacted]
MeOH - Extrakt
198 mg
LH-20-Trennung
LH-① LH-② LH-⑤ – LH-⑥
Verwerfen
82 mg
Epo A.3 + Epo neu
Verwerfen
RP-Trennung
RP-① RP-②
0,7 mg
NMR 002549
Epo neu
1,0 mg
NMR 00 2550, cosy
Epo neu
präp. DC
RP-① DC
0,4 mg
NMR 00 2630
Epo neu

Exhibit 3-15

15

EXHIBIT

DECLARATION OF TRANSLATOR

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R. Richards

Date: 5. August 2003

NMR REQUEST

GBF – Dept. of Molecular structure research

Date received: [redacted]
Spectrum no. 002630

Substance name: So 1198 – RP-1 / DC
Substance producer: Pohlaus
Dept.: NC (1.1-2) tel. 343
Nuclear species: $^{1}H_{1}$
Amount of substance : 0.4 mg
Suitable solvent: CD_3OD
Return substance? Yes

General Information
Store sample in fridge Y

Signal expected between
$\delta = 0$ and 9
Requested: only spectra Y
plus integral Y

Type of experiment

$^{1}H_{1}$ Standard spectrum Y

Plot and Data manipulation

$\delta = 8.9$ to — 0.1 (0.15 ppm/cm) Y

Filed under no. SIPR 2640\ ??

Einlieferungsdatum: [redacted]

Spektren-Nr.: 002630

[redacted]

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

Substanz-Bez.: So1198 IRP-①IDC

Summenformel: _____

Substanzhersteller: Roche

Abteilung: NC (1.1.2) Tel.: 343

Kernart (¹H, ¹³C, ³¹P, andere?) _____

Substanz-Menge: 0,4 mg, Molmasse: _____

geeignetes Lösungsmittel: CD3OD weitere Messung nach Zugabe von _____

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒

im Tiefkühlfach ☐

im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen

δ = 0 und 9

Gewünscht: nur Spektrum ☒

plus Integral ☒

Interpretation ☐

Zahl der Akkumulationen (falls > 104): _____

Art des Experiments

[¹H] Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

[¹³C] ¹H-Entkopplung:

Breitband ☐ selektiv ☐

DEPT ☐ ohne ☐

Entkoppler-Frequenz(en): _____

Plot und Datenmanipulation

Gauss-Multiplikation ☐

[¹H]

δ = 8.9 bis —0.1 (0.15 ppm/cm) ☒

11.9 bis —0.1 (0.2 ppm/cm) ☐

Linienausdruck ☐

Drehungen:

10 Hz/cm ☐ von δ = _____ bis _____

[¹³C] normal (δ = 220 bis 0) ☐

anderes Format: _____

Sonderwünsche: COSY ☐ ¹³C—¹H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☐ AM-300 ☐ ARX-400 ☐ DMX-600

gespeichert unter Nr. SIRR98010N _____, _____ _____, _____

Bitte um Rücksprache ☐

Kommentar:

_____

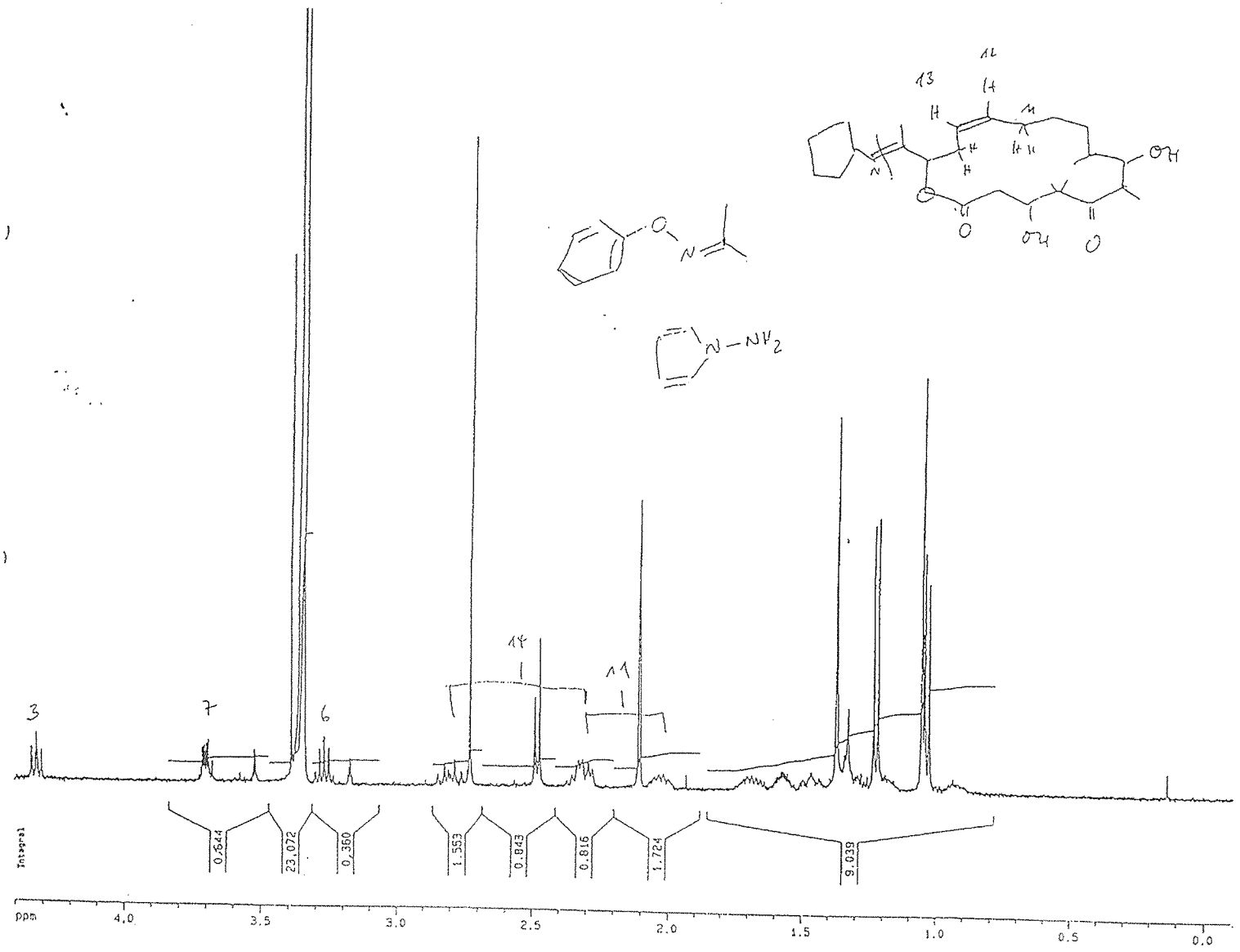
(Unterschrift)

So 1198-1
RP-① 1Dc

0,4mg

15

SIPR2630 10 1



Current Data Parameters

NAME	SIPR2630
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	11.39
INSTRUM	arx400
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	280
DS	2
SWH	8333.333 Hz
FIDRES	0.254313 Hz
AQ	1.9661300 sec
RG	4096
DW	60.000 usec
DE	85.71 usec
TE	300.0 K
D1	1.00000000 sec
P1	14.00 usec
DE	85.71 usec
SFO1	400.1324710 MHz
NUCLEUS	1H

F2 - Processing parameters

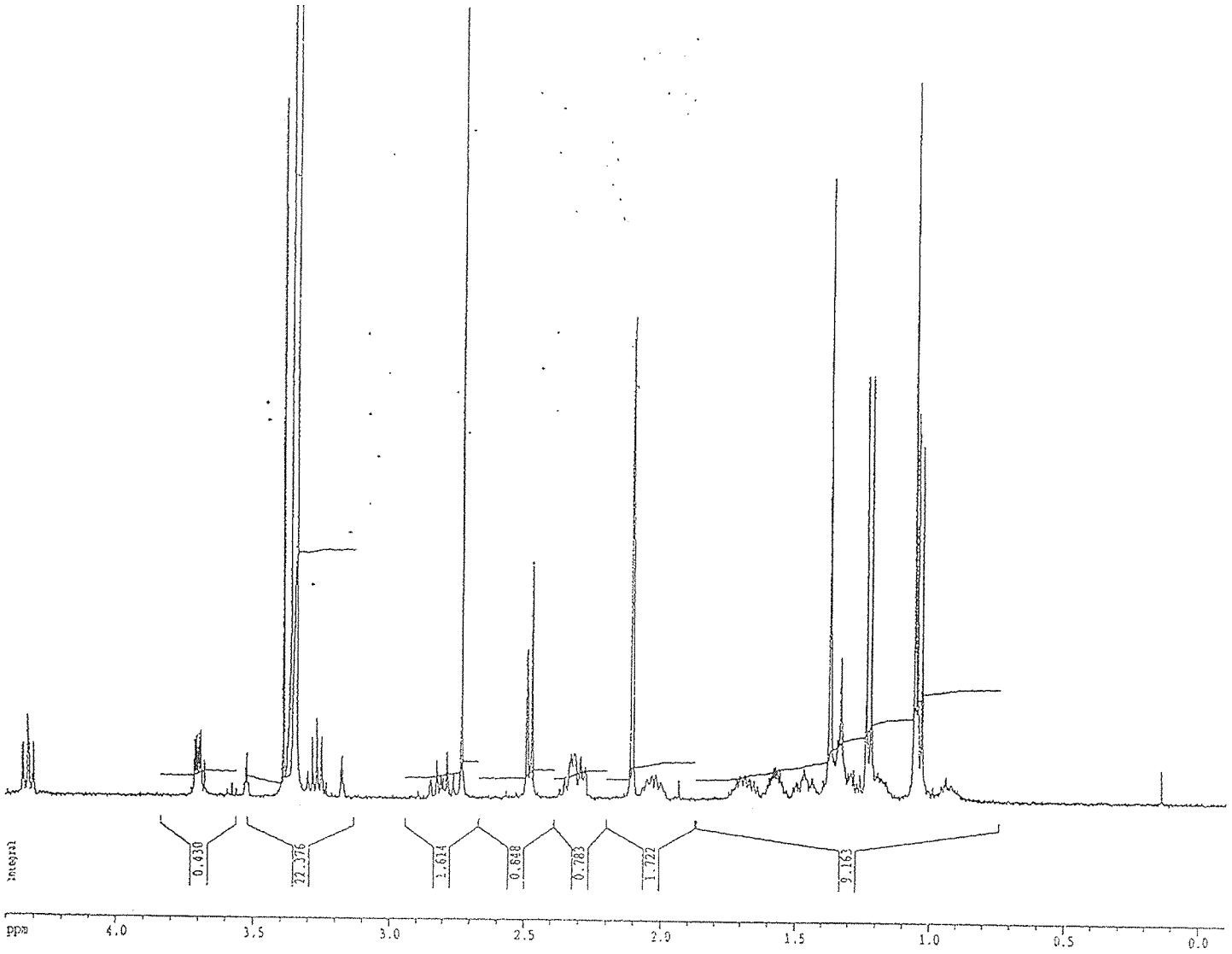
SI	16384
SF	400.1299912 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1760.57 Hz
F2P	-0.100 ppm
F2	-40.01 Hz
PPMCM	0,15000 ppm/cm
HZCM	60.01950 Hz/cm

Epo C
NMR Patent -
Approach

SIPR2630 10 1



Current Data Parameters

NAME	SIPR2630
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	11.39
INSTRUM	arx400
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	280
DS	2
SWH	8333.333 Hz
FIDRES	0.254313 Hz
AQ	1.9661300 sec
RG	4096
DW	60.000 usec
DE	65.71 usec
TE	300.0 K
D1	1.00000000 sec
P1	14.00 usec
DE 85.71 usec
SFO1 400.1324710 MHz
NUCLEUS 1H

20.6.96

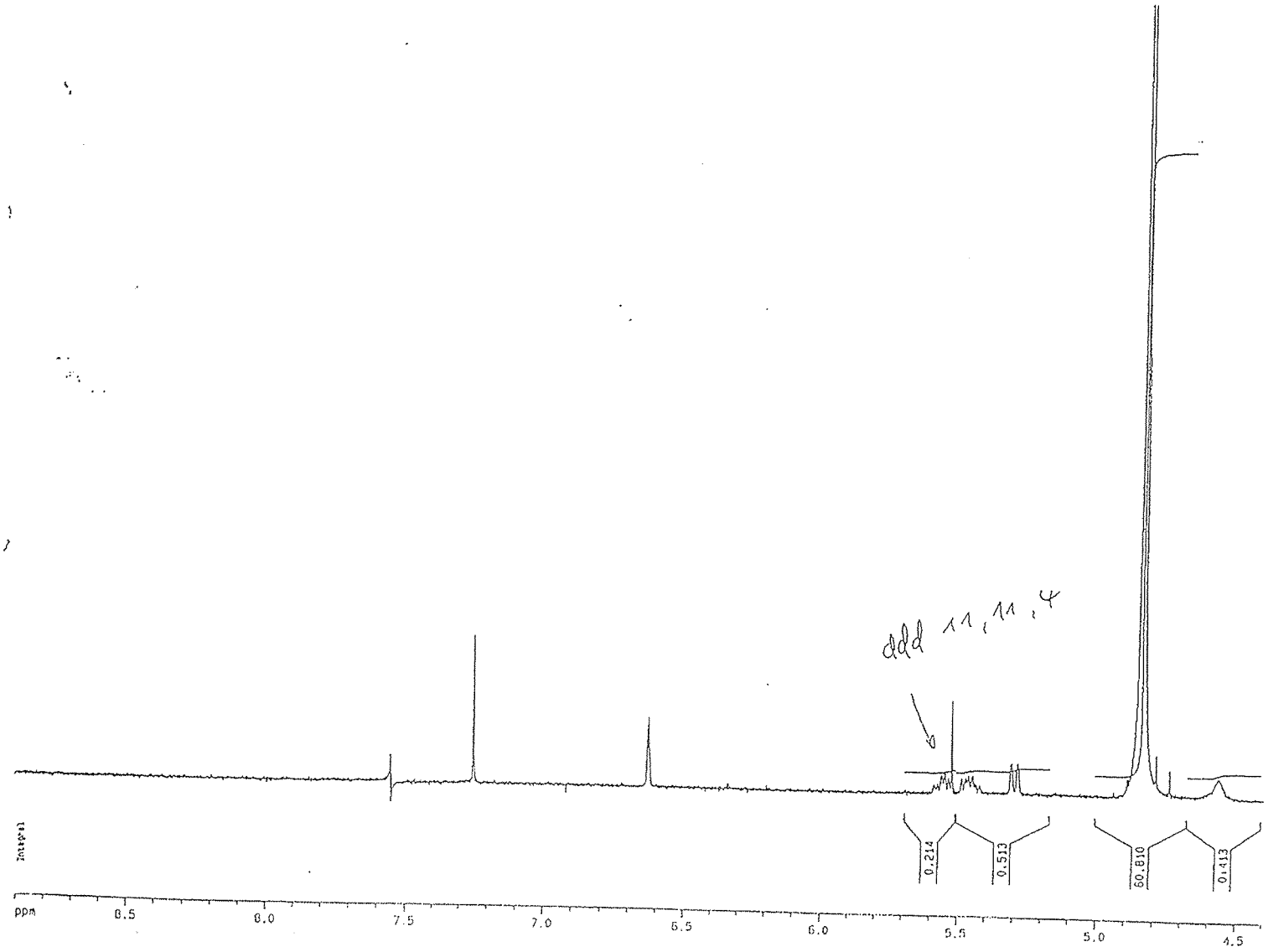
F2 - Processing parameters

SI	32768
SF	400.1299912 MHz
WDW	QSINE
SSB	2
LB	0.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1760.57 Hz
F2P	-0.100 ppm
F2	-40.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	60.01950 Hz/cm

SIPR2630 10 1



SIPR2630 10 1

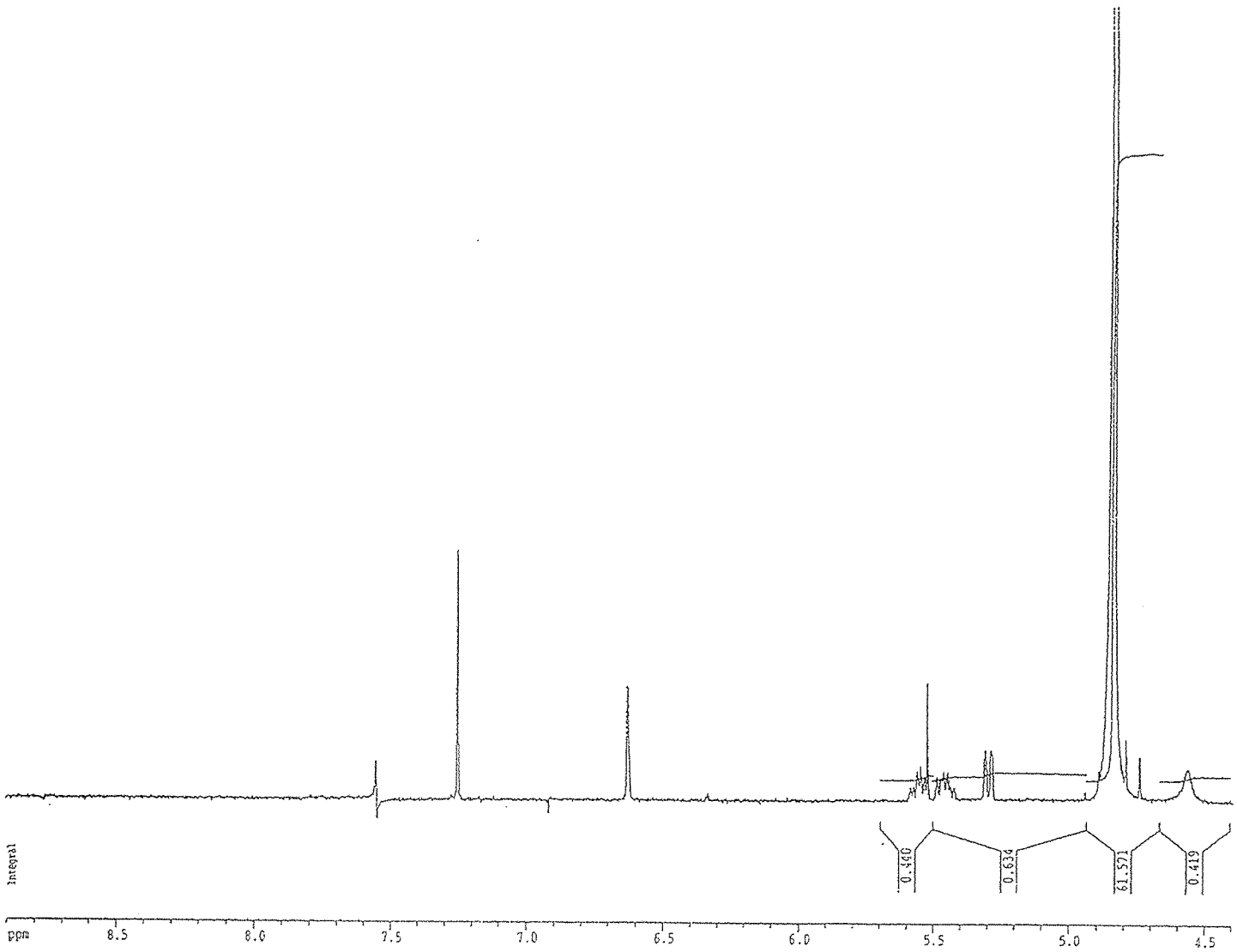


Exhibit 3-16

16

EXHIBIT

DECLARATION OF TRANSLATOR

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Date: 5. August 2003

Confidential

Minutes no. 231 of meeting held at 09.00 on [redacted]

Present: Frau Herrmann, Herr Augustiniak, Forche, Gerth, Höfle, Irschik, Jansen, Reichenbach, Sasse, Steinmetz, Washausen

Information:

- Two written offers of contract for epothilone have now been received, three more are expected in the near future.

- ASTRA has expressed an interest in testing etnangien in its polymerase tests. A test sample should be sent after concluding a confidentiality agreement.

- The NBI department plans to make TA culture extracts from myxobacteria available on payment to each of several firms for them to screen. GBF retains the rights to the strains and will have an appropriate share in any success.

- Rhone-Poulenc has applied for two patents covering a substance from actinomycete A 9738 (CBS 162.94), which is identical to Cittilin from Mx x48. The compound is described as a neurotensin antagonist. GBF's patent law firm is being asked to check whether these patents can't be challenged on the basis of our two publications and if necessary overturned.

- Ciba-Geigy Pharma in a letter dated [redacted] has released the following substances: eliamid, etnangien, argyrin A and B. A verbal indication suggested that chondramid might be released, since the substance has no in vivo effect.

- Myxothiazol must be given further medium-term fermentation (Kunze).

Epothilone (Gerth, Sasse, Steinmetz): In addition to the 15 known producers we already have, 9 were recently added from Herr Irschik's screening; eight of them formed Spirangien at the same time, one formed icumazol, one only formed epothilone A; productivity was not significantly high in any of the cases. A suitable production strain has to be selected from these known 24 producers. The strains So ce90 (the original producer), So ce660 (only forms

epothilone A), So ce950 (only forms icumazol), So ce1198 (free of extra substances) as well as So ce1275 and 1294 (both originate from the same sample, grow better, form no known extra substances) are at present being investigated in more detail (adaptation to homogenous growth, plating, clone selection). Tests on medium optimisation indicated that the different strains react in different ways. The addition of propionate with So ce90 caused increased formation of epothilone B; for the other strains this did not occur, synthesis in part even being totally blocked despite good growth (So ce1198, So e1275). The addition of formate to So ce90 caused increased formation of epothilone B and a reduction in epothilone A as well as increased synthesis overall; succinate on the other hand had no effect. So ce1198 and So ce1275 formed no epothilone at all with formate. The type of starch added also has dramatic effect on epothilone synthesis: for So ce90 the best results are achieved with Cerestar (100 %); the results with wheatmeal or ryemeal were considerably worse; using Ciba starch 37 % of the Cerestar yield was achieved, soluble starch achieved 74 %; with soluble starch the ratio of A to B shifted from 1.26 (Cerestar) to 0.83. For skimmed milk and yeast extract a quality comparison still needs to be carried out. Using complex substrates such as banana, plum, or mushroom flour resulted in good growth, but epothilone production was poor or even completely suppressed. So ce477 grew well in full-fat soya flour, So ce90 only grew in low-fat soya flour. While So ce90 did not produce anything on agar plates, So ce1198 still appeared to form epothilone on certain types of agar, which would make strain selection much easier. None of the strains have grown homogenously so far. Plating is possible with So ce1148: 50 clones have recently been isolated, of which 10 produce epothilone and 40 do not. The formation of Spirangien can easily be detected during cloning, so Spirangien producers can quickly be eliminated.

Fermentations of 9.8.-18.8.: F25 (10 l), starter culture: good growth, clean; transferred to F26 (100 l): good growth, clean; F27 (1000 l) had meanwhile been prepared: it frothed over and lost 450 l medium; the reqctor was filled again and autoclaved, but again lost another 80 l; it nonetheless remained sterile and was then inoculated from F27. An aliquot of 10 l was at the same time taken from F26 and inoculated into F28 (830 l): F27 and F28 were both infected with a bacillus after 1 day; it was discovered that the inoculation tube used for both inoculations had a hole. F29 (3000 l) was still planned however: after autoclaving the skimmed milk medium the reactor was unsterile after 1 day; it was then autoclaved again and was then still sterile after 4 days; the rest of the medium was then added: 2 days later the reactor was again unsterile and was autoclaved again, it was again unsterile shortly afterwards and was discarded. Since the Biotechnikum is totally closed from Week 37 to 40 due to the ITP, the next series of fermentations will only be possible from 20.9 to 10.10; it might be possible to

add a second cascade afterwards; it is planned to run with a total of 6340 l and 5400 l production volume. From Week 43 to 46 the brine plant is being repaired but this should not affect epothilone production. Due to frothing over of reactors and infections at early stages, 50 l of expensive XAD were lost.

At present there is about 600 mg epothilone A and 400 mg epothilone B available in very impure extracts and the material is being purified at great expense. The substance is urgently needed for test samples.

100 mg epothilone A and 100 mg epothilone B have recently been sold to Bristol-Myers, 150 mg A and 150 mg B to Boehringer.

A batch of about 1.5 g epothilone got lost during recovery. Following preparative HPLC the substance was still all right, it was rotated and left overnight; It was then chlorinated by adding HCl and was inactive.

The strains So ce1198, So ce1275 and So ce1294 form two new epothilones as well as epothilone, but with the epoxide missing. They had considerably reduced action, but were not inactive: The IC_{50} for L929 cells was 150 ng/ml for RP1 (from So ce1198), and 100 ng/ml for RP2. Noticeable effect on Tubulin could be detected in cell cultures. Perhaps patenting could be possible?

Epothilone had lost all activity in mouse serum after 4 h at 37°, and showed a similar result in rat serum; however, the substance was not inactivated in serum of humans, cattle, rabbits, goat or sheep (only serums that were not heat inactivated were used). In human serum the substance was completely stable for 2 days at 37° (HPLC analysis). Lyophilized mouse serums were inactive and hamster serum slightly active. Pig liver esterase opens the lactone ring.

Ambruticin (Gerth): Following feeding of ^{14}C Ambruticin A, only VS3 and S could subsequently be detected; radioactivity could not be found anywhere else.

From 2.9 Herr Dipl. Biol. Knauth will be working on the mechanism of action of ambruticin and jerangolid (NBI Dept.).

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Vertraulich

Protokoll Nr. 231 der Besprechung am [redacted] 9.00 Uhr

Teilnehmer: Frau Herrmann, die Herren Augustiniak, Forche, Gerth, Höfle, Irschik, Jansen, Reichenbach, Sasse, Steinmetz, Washausen

Zur Information:

- Für Epothilon liegen inzwischen zwei schriftliche Vertragsangebote vor, drei weitere sind in naher Zukunft zu erwarten.

- Die Firma ASTRA hat Interesse angemeldet, Etnangien in ihren Polymerase-Tests zu prüfen. Nach Abschluß eines Vertraulichkeitsabkommens soll ein Prüfmuster versandt werden.

- Die Abteilung NBI plant, an mehrere Firmen gegen Bezahlung von je einer TA Kulturextrakte von Myxobakterien für deren Screening zur Verfügung zu stellen. Die GBF behält die Rechte auf die Stämme und wird am Erfolg angemessen beteiligt.

- Rhône-Poulenc hat zwei Patente für eine Substanz aus Actinomycet A 9738 (CBS 162.94) angemeldet, die mit Cittilin aus Mx x48 identisch ist. Die Verbindung ist als Neurotensin-Antagonist beschrieben. Das Patentbüro der GBF wird gebeten zu prüfen, ob diese Patente nicht auf Basis unserer zwei Veröffentlichungen angegriffen werden können und diese dann ggf. auch zu kippen.

- Ciba-Geigy Pharma hat mit Schreiben vom [redacted] folgende Substanzen freigegeben: Eliamid, Etnangien, Argyrin A und B. Mündlich wurde eine Freigabe von Chondramid in Aussicht gestellt, da die Substanz in vivo nicht wirkt.

- Myxothiazol muß mittelfristig nachfermentiert werden (Kunze).

Epothilon (Gerth, Sasse, Steinmetz): Zu den 15 schon bekannten eigenen Produzenten kamen neuerdings 9 aus dem Screening von Herrn Irschik hinzu; von diesen bildeten 8 gleichzeitig Spirangien, einer Icumazol, einer nur Epothilon A; die Produktivität war in keinem Fall ungewöhnlich hoch. Aus den somit bekannten 24 Produzenten muß ein geeigneter

Produktionsstamm ausgewählt werden. Zur Zeit werden So ce90 (der ursprüngliche Produzent), So ce660 (bildet nur Epothilon A), So ce950 (bildet nur Icumazol), So ce1198 (frei von Begleitsubstanzen) sowie So ce1275 und 1294 (stammen beide aus derselben Bodenprobe, wachsen besser, bilden keine bekannten Begleitsubstanzen) näher charakterisiert (Adaption zu homogenem Wachstum, Plattierung, Klonselektion). Versuche zur Mediumsoptimierung zeigten, daß die einzelnen Stämme unterschiedlich reagieren. Zusatz von Propionat führt bei So ce90 zu einer verstärkten Bildung von Epothilon B; für die anderen Stämme gilt dies nicht, zum Teil wird die Synthese sogar trotz guten Wachstums total blockiert (So ce1198, So e1275). Zusatz von Formiat zu So ce90 führt zur verstärkten Bildung von Epothilon B und einer Reduzierung von Epothilon A sowie insgesamt zu einer verstärkten Synthese; Succinat hat dagegen keinen Effekt. So ce1198 und So ce1275 bilden mit Formiat überhaupt kein Epothilon. Auch die Art der zugesetzten Stärke beeinflußt die Epothilonsynthese dramatisch: Bei So ce90 werden die besten Ergebnisse mit Cerestar erhalten (100 %); mit Weizen- oder Roggenmehl Ergebnisse erheblich schlechter; mit Ciba-Stärke werden 37 %, mit löslicher Stärke 74 % der Ausbeute mit Cerestar erreicht; mit löslicher Stärke verschiebt sich dabei das Verhältnis von A zu B von 1.26 (Cerestar) zu 0.83. Für Magermilch und Hefeextrakt muß erst noch ein Qualitätsvergleich durchgeführt werden. Komplexe Substrate wie Bananen-, Pflaumen- oder Pilzmehl erhält man gutes Wachstum, aber die Epothilon-Produktion ist schlecht oder ganz unterdrückt. So ce477 wächst gut in Sojamehl vollfett, So ce90 dagegen nur in entfettetem Sojamehl. Während So ce90 auf Agarplatten nichts produziert, scheint So ce1198 auf bestimmten Agarsorten noch Epothilon zu bilden, was die Stammselektion sehr erleichtern würde. Keiner der Stämme wächst bisher homogen. So ce1148 kann plattiert werden: Inzwischen sind 50 Klone isoliert, von denen 10 Epothilon produzieren, 40 dagegen nicht. Die Bildung von Spirangien läßt sich beim Klonieren leicht erkennen, so daß Spirangien-Produzenten schnell ausgeschieden werden können.

Fermentationen vom 9.8.-18.8.: F25 (10 l), Starterkultur: gut gewachsen, sauber; überführt in F26 (100 l): gutes Wachstum, sauber; inzwischen war F27 (1000 l) vorbereitet worden: dieser schäumte aber über und verlor 450 l Medium; der Reaktor wurde wieder aufgefüllt und autoklaviert, verlor aber anschließend nochmals 80 l; trotzdem blieb er steril und wurde dann aus F27 angeimpft. Ein Aliquot von 10 l wurde aus F26 parallel in F28 (830 l) überimpft: F27 sowie F28 waren beide nach 1 d mit einem Bacillus infiziert; wie sich herausstellte, hatte der für beide Impfvorgänge verwendete Impfschlauch ein Loch. Außerdem war noch F29 (3000 l) geplant: Nach Autoklavieren des Magermilch-Mediums war der Reaktor nach 1 d unsteril; er wurde danach nochmals autoklaviert und war anschließend für 4 d steril; danach wurde der Rest des Mediums zugesetzt: 2 d später war der Reaktor wieder unsteril und wurde erneut autoklaviert, kurz danach war er wieder unsteril und wurde verworfen. Da das Biotechnikum in

der 37. - 40. Woche durch den ITP total blockiert ist, ist die nächste Fermentationsserie erst vom 20.9.-10.10. möglich; vielleicht läßt sich danach eine zweite Kaskade anschließen; vorgesehen ist insgesamt 6340 l Arbeitsvolumen mit 5400 l Produktionsvolumen. Von der 43.-46. Woche wird die Soleanlage repariert, was aber keine Auswirkungen für die Epothilon-Produktion haben sollte. Durch das Überschäumen der Reaktoren und die Infektionen auf frühem Stadium gingen 50 l teures XAD verloren.

Derzeit liegen in +/- stark verunreinigten Extrakten rund 600 mg Epothilon A und 400 mg Epothilon B vor und werden unter großem Aufwand gereinigt. Die Substanz wird dringend für Prüfmuster benötigt.

Vor kurzem wurden an Brystol-Myers 100 mg Epothilon A und 100 mg Epothilon B verkauft, an Boehringer 150 mg A und 150 mg B.

Eine Charge von rund 1.5 g Epothilon gingen bei der Aufarbeitung verloren. Nach präparativer HPLC war die Substanz noch in Ordnung, sie wurde einrotiert und stand über Nacht: Danach war sie durch HCl Addition chloriert und unwirksam.

Die Stämme So ce1198, So ce1275 und So ce1294 bilden neben Epothilon auch zwei neue Epothilone, denen das Epoxid fehlt. Deren Wirksamkeit war stark reduziert, jedoch nicht aufgehoben: Die IC_{50} für L929-Zellen betrug für RP1 (aus So ce1198) 150 ng/ml, für RP2 100 ng/ml. In Zellkulturen war auch eine deutliche Wirkung auf Tubulin zu erkennen. Vielleicht wäre eine Patentierung möglich?

Epothilon in Serum der Maus hatte nach 4 h bei 37° alle Aktivität verloren, ebenso in Serum der Ratte; in Serum von Mensch, Rind, Kaninchen, Ziege und Schaf wurde die Substanz dagegen nicht inaktiviert (verwendet wurden ausschließlich Seren, die nicht hitzeinaktiviert waren). In Humanserum war die Substanz über 2 d bei 37° völlig stabil (HPLC-Analytik). Lyophilisierte Seren der Maus waren unwirksam, des Hamsters etwas wirksam. Schweineleberesterase öffnet den Lactonring.

Ambruticin (Gerth): Nach Verfütterung von ^{14}C-Ambruticin A waren anschließend nur VS3 und S nachweisbar; nirgendwo sonst war Radioaktivität zu entdecken.

Ab 2.9. wird sich Herr Dipl.Biol. Knauth mit dem Wirkmechanismus von Ambruticin und Jerangolid beschäftigen (Abt. NBI).

Laborbuch Fischer

- Soce 1198 A5 (A37) in Flüssigkultur + auf Platte angeimpft ⟹
  ~~(konservieren 10 x + als Stammkultur weiter laufen lassen~~
  1198 A2 verwerfen (5x konservieren))

- F-Medium für 1 l
  Grundmedium:

  1,5 g MgSO4
  1 g Pepton (Merck) } pH 7,2
  1,4 g Hepes

- Stammlsg. herstellen
- Flaschen !!!
- Medium Fermenter !!! F 15 /
- Screening-Stämme weiterimpfen (3x250 ml): 1332, 1335, 1338, 1339
- E-Medium für 1,2 l (Protokoll 89 + 90)

  4,8 g – Magermilch
  2,4 g – Yeast extrakt
  12 g – Stärke
  1,2 g – $CaCl_2$
  1,2 g – $MgSO_4$
  14,3 g – Hepes
  1,2 ml – Fe-EDTA
  4,8 g – Sojamehl
  6 g (ml) – Glycerin
  } pH 7,4 + 2 ml XAD / Kolben

- Protokoll 88 + Vorbereitung P 89 + P90 auch

/ Mi, [redacted] /

- Protokoll 88, 89 + 90 animpfen
- Soce 90 A3 weitergeimpft für Fermentation (4x250 ml)
- Erute von Protokoll 77 + DSM-Stamm
- Stammlsg. herstellen:

Protokoll 89 : • 10 %-ige Propionatlsg. = 10 g Propionat + 90 ml H_2O
Protokoll 90 : • 10 %-ige Formiatlsg = 10 g Formiat + 90 ml H_2O
Med. F : • Stammlsg 1 : 20 % KNO_3 = 20 g KNO_3 + ~~50 ml H_2O~~
1,25 % K_2HPO_4 = 1,25 g K_2HPO_4 } + 78,75 ml H_2O
• Stammlsg - 2 : 10 % $CaCl_2$ = 10 g $CaCl_2$
80 mg/l Fe/EDTA = 80 mg Fe/EDTA } + 89,92 ml H_2O
• Stammlsg - 3 : 25 % Glukose = 25 g Glukose + 75 ml H_2O

- 35 %-ige Glukose : 70 g Glukose + 130 ml H_2O
Ca2-Agar • Stammlsg 1 : 15,0 g KNO_3, 15,0 g K_2HPO_4 + 180 ml H_2O
• Stammlsg 2 : 2,0 g $MgSO_4$ + 196,0 ml H_2O
• Stammlsg 3 : 0,4 g $CaCl_2$ + 0,25 g $FeCl_3$ + 199,35 ml H_2O

⟹ PROPIONAT ? ⟸

| [redacted]

4-11

- 10 Kolben 1198 A2 ernten: XAD zu Hr. Steinmetz
- Glukose 25% + 35%ig [redacted] steril filtrieren
- E-Medium für 5 l

20 g - Magermilch
20 g - Soyamehl
10 g - Yeast extrakt
50 g - Stärke
5 g - $CaCl_2$
5 g - $MgSO_4$
59,5 g - Hepes
5 ml - Fe-EDTA

} pH 7.4.

20 Kolben á 250 ml + 10 ml XAD

- F-Medium für 2 l
  Grundmedium

5 g - $MgSO_4$
2 g - Pepton (Marcor)
23,8 g - Hepes

} pH 7.2

20 Kolben á 100 ml + 2 ml XAD

→ wenn das Medium erkaltet ist, die Stammlsg. + Glukose dazugeben ←

/ Fr. [redacted]

- Stammkulturen überimpfen
- 12 x 250 ml Soce 90 A3 → Fermenter
- Versuch 10 x 500 ml mit 1198 A2 angeimpft
- Zugabe von Pro-produkt + Fermentat bei den Protokollen 89 + 90
- Ernte von Protokoll 86

/ Mo, [redacted]

- Stammkulturen überimpfen
- 1 l von Ferm. Soce 90 A3 weiterimpfen
- Screening-Stämme weiterimpfen (1 x 250 ml) Soce 1241 + 1270
  Stamm Soce 1305 verworfen → wächst nicht!
- Ernte der Screening-Stämme Soce 1325 + 1328
- Protokoll 77 + 86 einengen + HPLC
- Im Lager Medium abwiegen (F 150 2x [illegible]) Dr. Firth selber
- Klone von Soce 90 ernten (148 Stck.)
- Fr. F-Medium mit Soce 90 A3, 1275 + 1198 A5 ⇒ Fr. 27.9. (nach 1 Woche HPLC Überprüfung) animpfen.
- Konservierung -71°C Soce 90 A3 10x, Soce 1198 A2 10x
- 50 Kolben á 20 ml E-Medium kochen (1 l)

/ Di, [redacted]

- 50 Kolben (100 ml) á 20 ml E-Medium + XAD (0,5 ml) (1 l Medium)
- restliche Medien für Fermentation abwiegen + ins Technikum bringen (Flaschen + XAD ebenfalls)
- F 15 (2x) animpfen → parallel (10^15 Uhr)
- Ernte: Screening-Stämme Soce 1333

⇒ Biologisches Screening! ⇐

4-12

E-Medium für 1 l

4 g – Magermilch
4 g – Soyamehl, entfettet
2 g – Yeast extrakt
10 g – Stärke
1 g – $CaCl_2$
1 g – $MgSO_4$
11,9 g – Hepes
1 ml – Fe-EDTA

pH 7,4

50 Kolben à 20 ml + 0,5 ml XAD

Ernte von Protokoll 88, 89, 90
E-Medium animpfen mit 9043, 1198 A5 + 1275 je 5 Kolben

Soce 1198 A5 10 x 2 ml bei -71 °C konservieren
E-Medium animpfen mit Soce 9043 1275 + 1198 A5 je 5 Kolben (Zusätze nicht vergessen beim Medium) ⟹
nach 1 Woche HPLC-Überprüfung ✓ e- pr. pH
Biologisches Screening (22 Stämme) ✓

[redacted] – [redacted]

Ernte → Stirnhöhlenvereiterung

110, 07.10.96

Soce 1198 A5 + 1300 10 x 2 ml tous. -71 °C
Ernte von Screening-Stämmen Soce 1334, 1336, 1332, 1335, 1338 + 1339
Soce 1328 schon in 50 ml
Screening-Stämme Soce 1271 + 1270 weiterimpfen in 3 x 250 ml
Versuch mit 1198 A2 ein Kolben ernten + Analytik erstellen
Screening-Stämme neu animpfen: Soce 1308, 1342, 1349, 1352, 1364
MIC-Bestimmung für Oliver Frohwald 6 Stck.
600 ml E-Medium kochen für Protokoll 91 (Soce 9043 ansetzen)

Konzentrationsbestimmung von Epothilon

Fläche x 4 x 0,0007072 µg
= Fläche x 0,0028288
(Area [mAU*s])

FERMENTER: Blatt 1 Kostenstelle: 103310 Vers.Nr.: 96/145/02/02

Betreiber: K. Sulk **Betreuer:** M. Hacker

Organismus: Soce 90

Kulturführung: Aerob: o Anaerob: o Phototroph: o

Prozeßführung: Batch: o Feed-Batch: o Konti: o

Fermenteraufbau:

Fermenter Nr. 15 L 150 L	Verwendung: Fermentation ~~o~~ Vorlage o	Steriltest o für Protokoll-Nr. __/__
Sicherheitsmaßnahmen	Abluftfilter: Nein ~~o~~ Ja o Handschuhe tragen: Nein o Ja ~~o~~	
Betrieb-Beginn	Datum: [redacted]	Uhrzeit: 8⁰⁰
Rührerart	3 x Scheibe	
Sondergeräte		
Pumpe für —	Typ: —	Pumprate: — Durchm$_{Schlauch}$: —
Pumpe für —	Typ: —	Pumprate: — Durchm$_{Schlauch}$: —
	Medium mit Antischaum	

Elektroden:

pH-Elektrode	Nr.: 8	Puffer 1: 4,0 Poti/ mV: —	Puffer 2: 4,0 Poti/ mV: —
pH-Elektrode	Nr.:	Puffer 1: Poti/ mV:	Puffer 2: Poti/ mV:
pO_2-Elektrode	Nr.: 02013	Nr.:	

Reaktorgewicht: Gesamtgewicht

Sollgewicht	82	[KG]
leer	—	[KG]
Wassermenge	44,52 [l]	[KG]
Medium-Zugabe	Name: Soce-90 Herk.: Nutzer ~~o~~ SE: __/__	[KG]
	XAD Zugabe: ~~o~~ Ja o Nein	
Antischaum	Art: Desaro Volumen: 30 [ml]	
pH vor Sterilisation	Ist: 6,25 Soll: 7,0	
pH eingestellt mit	Name: KOH Konz.: 5 N Menge: 13 ~~14~~ ml	

Sterilisation:

Steril. Gleitringdichtung	Datum:	Uhrzeit:	Dauer:	min
1. Sterilisation Fermenter	Datum: [redacted]	Uhrzeit: 8⁰⁰	Dauer: 60	min
2. Sterilisation Fermenter	Datum:	Uhrzeit:	Dauer:	min
pH nach Sterilisation	4,0	Reaktorgewicht nach St.	82 kg	

Fermenter: Blatt 3 Kostenstelle: 103310 Vers.Nr.: 96/145/02/02

Inokulation:

Inokulum 1	Herk.: Nutzer ~~0~~ Protokoll-Nr.: __/__ Flasche Nr. 05/10	Volumen: 1 [l]
Fermentation Beginn	Datum: [redacted] Uhrzeit: 10 [illegible]	
Inokulum 2	Herk.: Nutzer 0 Protokoll-Nr.: __/__ Flasche Nr. ____	Volumen: [l]
Inokulum 2 Zeitpunkt	Datum: Uhrzeit:	
Fermentergewicht nach	Inokulierung 1: 10 [kG]	Inokulierung 2: [kG]

Fermentation-Ende:

Fermentation-Ende	Datum: [redacted] Uhrzeit: 1425	
Fermenter-Gewicht	12	
Korrekturmittel: Volumen nach der Fermentation	Säure 1: / Lauge 1: Antischaum 1:	Säure 2: / Lauge 2: Antischaum 2: / Säure 3: Lauge 3:
Volumen nach Ferm. von	Zufütterung 1:	Zufütterung 2:
wie geplant 0		
Kontamination o	Zeitpunkt: Vor den Animpfen o Nach dem Animpfen o	Vorkultur o
Defekt o	Art:	
übergeschäumt o	Zeitpunkt: Sterilisation: Aufheizphase o Haltephase o Abkühlphase o	Kultivierung: Vor den Animpfen o während der Kultivierung o am Ende der Kultivierung o
sonstiges		

Weiterverarbeitung:

Transferleitung Sterilisat.	Datum:		Uhrzeit:		Dauer:			
Ablassleitung Sterilisat.	Datum: [redacted]		Uhrzeit: 17 [illegible]		Dauer: 120			
Nächster Schritt der Weiterverarbeiung:	Aufarbeitung 0 An Nutzer übergeben 0		Übergeimpft auf einen Fermenter 0 Übergeimpft auf mehrere Fermenter 0					
Volumen [l]	52							
Protokoll-Nr. der nächsten Schritte	02/05	__/__	__/__	__/__	__/__	__/__	__/__	__/__

Entsorgung:

Sterilisation Abluftfilter	Datum: Zeit: Dauer:
Inaktivierung Fermenter	gesamter Inhalt o restl. Inhalt o Vol:___ [l] Überstand o
	Datum: 30.08.96 Uhrzeit: 800 Dauer: 5 Temp.: 80°
Besonderheiten	[illegible]
Betriebs-Ende	Datum: 30.08.96 Uhrzeit: 1120

Anmerkungen/Besonderheiten zur Fermentation: Medium mit Ultraturrax suspendiert

30 ml Tegospon

Aufarbeitung

Zielsetzung:

Feststoffabtrennung:

o Zentrifugation o Mikrofiltration o Dead-end-Filtration

o Adsorberharzabtrennung

benötigt werden ---> o Filtrat/Überstand o Feststoff

o Lyophilisation o Ultrafiltration

o Verdampfung gewünschtes Endvolumen :.....(L/ml)

max.Temp. :.....(°C)

Extraktion:

o Kulturbrühe o Überstand o Feststoff

Verteilungskoeffizient:

Lösungsmittel/Zusätze:

Phasenverhältnis: Stufenzahl:

Zusatzprotokolle:

Produktspezifische Besonderheiten/weitergehende Aufarbeitungsschritte/Analytik:

Toxische Eigenschaften/Sicherheitsmaßnahmen:

Besonderheiten der Entsorgung/Dekontamination von Mikroorganismen bzw. toxischen Produkten:

ACHTUNG!! Lagerzeiten von Kühlgut max.3 Arbeitstagen, von Gefriergut max.3 Monate!! Nach Terminüberschreitung erfolgt Entsorgung!!)

Datum/Unterschrift: [redacted]

4-16

Wägeprotokoll F 15/1

Versuchsnummer:
175/02/02

Mikroorganismus:
Soce 90

Code für Medium:
E - Medium

Behälterbeschriftung:

1	
2	
3	
4	

Nr.	Substanz	Firma	Nr. / batch	Soll	Einwaage	kg / g / mg / l
1	Magermilch			40 g	40,02	g
2	Yeast extrakt	Ciba		20 g	20,00	g
3	Stärke			100 g	100,01	g
4	CaCl2			10 g	10,02	g
5	MgSO4			10 g	10,00	g
6	Fe-EDTA			80 mg	80,00	mg
7	Hepes			119 g	119,01	g
	* 0,5 l H2O					

Datum:
[redacted]

Unterschrift:
C. Fiscb

Hinweise: Die Versuchsnummer wird bei Abgabe des Anmeldeprotokolls vergeben / Mediumscode und Bezeichnung des Mikroorganismus wie im Anmeldeprotokoll / das Protokoll kann für mehrere Behälter benutzt werden, die Nr. in der ersten Spalte ist die Behälternummer (siehe 'Behälterbeschriftung') / bei Lösungen unter Substanz z.B. Wasser aufführen und Menge in ml oder l angeben / werden Stammlösungen verwendet, ist deren Zusammensetzung beizufügen

4-16

4-17

Verlaufs-Protokoll: Ferm.Nr.: _____ Kostenstelle: _____ Vers.Nr.: 96/____/02/__

Organismus: _____

Betreiber: _____

Datum	Zeit	Probe Vol.						Zugabe	Para-meter	Wert alt	Wert neu	Bemerkungen
[redacted]	15[10]	50										
[redacted]	8[15]	100										
[redacted]	8[15]	100										
[redacted]	8[05]	100										
[redacted]	8[05]	50										

4-17

Fermentations- und Aufarbeitungsprotokoll

Technikumsleitung	:	Dr. A. Roß	Tel. 130	priv.: (0 53 1)	34 35 49
Fermentation	:	H. Schüler	Tel. 131	priv.: (0 53 71)	79 48
Aufarbeitung	:	R. Krützfeldt	Tel. 137	priv.: (0 53 02)	45 62
Technik	:	10332	Tel.	priv.: ()	

Auftragsnummer: [redacted] Reaktor-Nr: [redacted] Anmeldedatum: .. .. ..

Name: Fischer ..... Berichtsfirma: NBI

Dienstteldon: 465 Privatteldoa:

Stamm/Medium: Soca 40 o Stammanmeldung liegt vor / ✗ liegt bei

Zid: Vorfermenter

Prozessbeginn am: [redacted] um 10 15 Uhr Prozessende am [redacted] um 10 00 Uhr

Startvolumen: 10 l Volumen der Vorkultur + sonst. Zugaben: 1 l

Vorkultur: Schüttelkultur ✗ Anforderer o Biotechnikum o Reaktor/Exp.Nr.

Medium: Nr. E ✗ trocken o gelöst ca. kg/l ✗ AS

o wird vom Biotechnikum angesetzt o wird geliefert von am

Vorlage	Art	Anhalt	Konz.	Menge (l oder kg)	Pumpentyp	Rate (max.)
1						
2						
3						
4						
5						

Waagen o 1 o 2 o 3 o 4 o 5 Timer o 1 o 2 o 3 o 4 o 5

o pH-Einstellung vor Sterilisation auf 7,0 mit ca. ml/l KOH

Sterilisation bei 121°C für min / Kalibrieren h / Sterilisat h

Startwerte für die Kultivierung:

Temperatur: 30 °C Belüftung: 0,1 vvm Nm^3/h

Drehzahl: 150 rpm pH-Wert: > 7,0 <

Druck: mbar

pO2-Messung o nein ✗ ja

Abgasmessung o nein o ja, Kanal

pO2-Regelung o nein o ja, Sollwert % Sättigung

Druckregelung o nein o ja, Sollwert mbar

Rechnererfassung o nein o ja, Exp.Nr.

GVO o ja

Parameter ändern

nach h --> nach h -->

nach h --> nach h -->

nach h --> nach h -->

weitere Angaben siehe umseitig ------>

Das Fermentationsprotokoll sollte in der der Fermentation vorangehenden Kalenderwoche bereits vorliegen, spätestens jedoch zwei Tage vor Beginn der Fermentation. Mündliche Fermenterreservierungen werden nur bis zu diesem Zeitpunkt berücksichtigt.
Der Nutzer verpflichtet sich, nicht abgesprochene Manipulationen an Geräten zu unterlassen und im Technikum die Sicherheitsvorschriften (z.B. UVV 102) einzuhalten.

Substrat und Hilfsmittel Zugabe:(nach Sterilisation)

Art	Herkunft	Vol. [ml]	Datum	Zeit	$Gew._{gesamt}$(KG)
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				

Vorlagen und Korrekturmittel:

Lauge 1:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Lauge 2:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Lauge 3:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 1:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 2:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 3:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Antischaum 1:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Antischaum 2:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 1 Art:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 2 Art:	$Vol._{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____

Regelung und Fermentationsstrategie, Startwerte:

pH-Sollwert: 7,0	eingestellt mit :		pH-Regelung von ..... bis .....
pO_2	Messung nein o	ja x	Regelung nein x ja o
pO_2 Sollwert:	Strategie: Drehzahl o	Zuluft o	sonstige o ____
Temperatur 30 [°C]	Druck [mbar]		Drehzahl 150 [rpm]
Parameter:	Sollwert: [.....]		Strategie:
Parameter:	Sollwert: [.....]		Strategie:
Parameter:	Sollwert: [.....]		Strategie:
Begasung: Luft andere: Sauerstoff ____ ____	____ l/min ____ l/min ____ l/min ____ l/min		Optimum = 60.1 l/h vvm ____ vvm ____ vvm ____ vvm
Überlagerung GLRD	Luft o	Dampf o	
Abgasmessung	nein x	ja o	Kanal: Kanal C
Rechnererfassung nein o	Exp.: 26152	Start-Datum: [redacted]	Zeit:

Fermentations- und Aufarbeitungsprotokoll

4-20

Technikumsleitung	:	Dr. A. Roß	Tel. 130	priv.: (0 53 1)	34 35 49
Fermentation	:	H. Schäfer	Tel. 131	priv.: (0 53 71)	79 48
Aufarbeitung	:	R. Krüger	Tel. 137	priv.: (0 53 07)	45 62
Technik	:	1033/10	Tel. -	priv.: ()	

Auftragsnummer: [redacted] Reaktor-Nr: 1.2 Anmeldedatum: 00.00.00

Name: Gerth / Fischer Berichtsform: NBI

Durchwahl: 433 / 465 Privatwahl:

Stamm/Medium: Soce 90 o Stammanmeldung liegt vor / x liegt bei

Ziel: Vorfermenter o Produktion von Epothilon (wenn anderer Stamm)

Prozeßbeginn am: [redacted] um 10:- Uhr Prozeßende am [redacted] um 10:- Uhr

Startvolumen: 100 l Volumen der Vorkultur + sonst. Zugaben: 10 l

Vorkultur: Schüttelkultur o Anforderer o Biotechnikum o Reaktor/ Exp.Nr.

Medium: Nr. E x trocken o gelöst ca. kg / l x AS

o wird vom Biotechnikum angesetzt o wird geliefert von am

Vorlage	Art	Anzahl	/ Konz.	/ Menge(l oder kg)	/Pumpentyp	/Rate (max.)
1	Lauge	KOH	10 %			
2	A.S.	Tegosipon				
3						
4						
5						

Waagen o 1 o 2 o 3 o 4 o 5 Timer o 1 o 2 o 3 o 4 o 5

o pH-Einstellung vor Sterilisation auf 7,6 mit ca. ml/l KOH

Sterilisation bei 121°C für min / Inaktivieren h / Sterilteil h

Startwerte für die Kultivierung

Temperatur:	30 °C	Belüftung:	0,1 vvm Nm³/h
Drehzahl:	200 rpm	pH-Wert:	x 7,0 konstant
Druck:	mbar		

pO2-Messung	o nein	x ja	
Abgasmessung	o nein	o ja, Kanal	
pO2-Regelung	o nein	o ja, Sollwert	% Sättigung
Druckregelung	o nein	o ja, Sollwert	mbar
Rechnererfassung	o nein	o ja, Exp.Nr.	

GVO o ja

Parameter ändern

nach h --> nach h -->

nach h --> nach h -->

nach h --> nach h -->

weitere Angaben siehe umseitig ---------->

Das Fermentationsprotokoll sollte in der der Fermentation vorangehenden Kalenderwoche freitags vorliegen, spätestens jedoch zwei Tage vor Beginn der Fermentation. Mündliche Fermentationsanweisungen werden nur bis zu diesem Zeitpunkt berücksichtigt.
Der Nutzer verpflichtet sich, nicht abgesprochene Manipulationen an Geräten zu unterlassen und im Technikum die Sicherheitsvorschriften (z.B. UVV 102) einzuhalten.

Anmerkungen/Besonderheiten zur Fermentation: Medium mit Ultraturrax suspendieren, 70 ml Tegospan i. HAD-Zugabe

Aufarbeitung

Zielsetzung: evtl.

Feststoffabtrennung:

o Zentrifugation o Mikrofiltration o Dead-end-Filtration

o Adsorberharzabtrennung

benötigt werden ---> o Filtrat/Überstand o Feststoff

o Lyophilisation o Ultrafiltration

Verdampfung gewünschtes Endvolumen :.....(L/ml)

max.Temp. :.....(°C)

Extraktion:

o Kulturbrühe o Überstand o Feststoff

Verteilungskoeffizient:.....

Lösungsmittel/Zusätze:.....

Phasenverhältnis:..... Stufenzahl:.....

Zusatzprotokolle:.....

Produktspezifische Besonderheiten/weitergehende Aufarbeitungsschritte/Analytik:

Toxische Eigenschaften/Sicherheitsmaßnahmen:.....

Besonderheiten der Entsorgung/Dekontamination von Mikroorganismen bzw. toxischen Produkten:

ACHTUNG!! Lagerzeiten von Kühlgut max.3 Arbeitstagen, von Gefriergut max.3 Monaten! Nach Terminüberschreitung erfolgt Entsorgung(!)

Datum/Unterschrift: [redacted] C. Fischer

4-22

Wägeprotokoll F 150/2

Versuchsnummer:
14510?/03

Mikroorganismus:
Soce 90

Code für Medium:
E-Medium

Behälterbeschriftung:

1	
2	
3	
4	

Datum:
[redacted]

Unterschrift:
C. Tisch

Nr.	Substanz	Firma	Nr. / batch	Soll	Einwaage	kg / g / mg / l
1	Magermilch			400 g	400	g
2	Yeast extrakt	Giba		200 g	200	g
3	Stärke			1 kg	1	kg
4	$CaCl_2$			100 g	100,02	g
5	$MgSO_4$			100 g	100,05	g
6	Fe-EDTA			800 mg	804,0	mg

Hinweise: Die Versuchsnummer wird bei Abgabe des Anmeldeprotokolls vergeben / Mediumscode und Bezeichnung des Mikroorganismus wie im Anmeldeprotokoll / das Protokoll kann für mehrere Behälter benutzt werden, die Nr. in der ersten Spalte ist die Behälternummer (siehe 'Behälterbeschriftung') / bei Lösungen unter Substanz z.B. Wasser aufführen und Menge in ml oder l angeben / werden Stammlösungen verwendet, ist deren Zusammensetzung beizufügen

93.9.

4-23

FERMENTER: Blatt 1 Kostenstelle: 203310 Vers.Nr.: 96/1455702/03

Betreiber: M. Jutta **Betreuer:** Sterlinski

Organismus: Sac 90

Kulturführung: Aerob: o Anaerob: o Phototroph: o

Prozeßführung: Batch: o Feed-Batch: o Konti: o

Fermenteraufbau:

Fermenter Nr. 170.2	Verwendung: Fermentation x Vorlage o	Steriltest o für Protokoll-Nr. __/__
Sicherheitsmaßnahmen	Abluftfilter: Nein o Ja o Handschuhe tragen: Nein o Ja x	
Betrieb-Beginn	Datum: [redacted]	Uhrzeit: 1500
Rührerart	3x Scheib	
Sondergeräte		
Pumpe für Lauge	Typ: Flocon 5001 Pumprate:	Durchm$_{Schlauch}$:
Pumpe für	Typ: Pumprate:	Durchm$_{Schlauch}$:
	Medien mit Hefe kurz	

Elektroden:

pH-Elektrode	Nr.: 2007	Puffer 1: 7 Poti/ mV: 5,16	Puffer 2: 4 Poti/ mV: 280
pH-Elektrode	Nr.:	Puffer 1: Poti/ mV:	Puffer 2: Poti/ mV:
pO_2-Elektrode	Nr.: 500.7	Nr.: 520,9	

Reaktorgewicht:

		Gesamtgewicht
Sollgewicht	90.2	[KG]
leer		-5 [KG]
Wassermenge	68 [l]	63 [KG]
Medium-Zugabe	Name: Herk.: Nutzer 0 SE: 02/02	84 [KG]
	XAD Zugabe: x Ja o Nein	-82
Antischaum	Art: Desmophen Volumen: 20 [ml]	
pH vor Sterilisation	Ist: 6,82 Soll: 7.0	
pH eingestellt mit	Name: 12 M Konz.: 5 N Menge: 30 ml	

Sterilisation:

Steril. Gleitringdichtung	Datum: [redacted]	Uhrzeit: 935	Dauer: 45	min
1. Sterilisation Fermenter	Datum: [redacted]	Uhrzeit: 1220	Dauer: 60	min
2. Sterilisation Fermenter	Datum:	Uhrzeit:	Dauer:	min
pH nach Sterilisation	7,08	Reaktorgewicht nach St.	82 kg	

Substrat und Hilfsmittel Zugabe:(nach Sterilisation)

Art	Herkunft	Vol. [ml]	Datum	Zeit	Gew.$_{gesamt}$(KG)
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				

Vorlagen und Korrekturmittel:

Lauge 1: NaOH 10%	Vol.$_{Anfang}$: 1500 Dat./Zeit: 27.9./1012	Herk.: Flasche Nr. 533
Lauge 2:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Lauge 3:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 1:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 2:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 3:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Antischaum 1: Tegosipon	Vol.$_{Anfang}$: 500 Dat./Zeit: 27.9./1012	Herk.: Flasche Nr. 554
Antischaum 2:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 1 Art:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 2 Art:	Vol.$_{Anfang}$: Dat./Zeit:	Herk.: Flasche Nr. ____

Regelung und Fermentationsstrategie, Startwerte:

pH-Sollwert:	eingestellt mit :	pH-Regelung von 7.0 bis .....
pO_2	Messung nein o ja ☒	Regelung nein ☒ ja o
pO_2 Sollwert:	Strategie: Drehzahl o Zuluft o sonstige o ____	
Temperatur 30 [°C]	Druck [mbar]	Drehzahl 200 [rpm]
Parameter:	Sollwert: [.....]	Strategie:
Parameter:	Sollwert: [.....]	Strategie:
Parameter:	Sollwert: [.....]	Strategie:
Begasung: Luft andere: ____ ____ ____	10 l/min ____ l/min ____ l/min ____ l/min	0,1 vvm ____ vvm ____ vvm ____ vvm
Überlagerung GLRD	Luft ☒ Dampf o	
Abgasmessung	nein o ja ☒	Kanal: 2
Rechnererfassung nein o	Exp.: AG1453 Start-Datum: [redacted]	Zeit: 1000

Fermenter: Blatt 3 Kostenstelle: 203310 Vers.Nr.: 96/145/02/03

Inokulation:

Inokulum 1	Herk.: Nutzer 0 Protokoll-Nr.: 02/04 Flasche Nr. 592	Volumen: 9 [l]
Fermentation Beginn	Datum: [redacted] Uhrzeit: 1025	
Inokulum 2	Herk.: Nutzer 0 Protokoll-Nr.: __/__ Flasche Nr. ____	Volumen: [l]
Inokulum 2 Zeitpunkt	Datum: Uhrzeit:	
Fermentergewicht nach	Inokulierung 1: 91 [kG]	Inokulierung 2: [kG]

Fermentation-Ende:

Fermentation-Ende	Datum: [redacted] Uhrzeit: 1310		
Fermenter-Gewicht	90kg		
Korrekturmittel: Volumen nach der Fermentation	Säure 1: Lauge 1: 1400 Antischaum 1:	Säure 2: Lauge 2: Antischaum 2:	Säure 3: Lauge 3:
Volumen nach Ferm. von	Zufütterung 1:	Zufütterung 2:	
wie geplant			
Kontamination o	Zeitpunkt: Vor den Animpfen o Nach dem Animpfen o	Vorkultur o	
Defekt o	Art:		
übergeschäumt o	Zeitpunkt: Sterilisation: Aufheizphase o Haltephase o Abkühlphase o	Kultivierung: Vor den Animpfen o während der Kultivierung o am Ende der Kultivierung o	
sonstiges			

Weiterverarbeitung:

Transferleitung Sterilisat.	Datum: [redacted] Uhrzeit: 1100 Dauer: 120 min							
Ablassleitung Sterilisat.	Datum: Uhrzeit: Dauer:							
Nächster Schritt der Weiterverarbeiung:	Aufarbeitung 0 An Nutzer übergeben 0			Übergeimpft auf einen Fermenter 0 Übergeimpft auf mehrere Fermenter 0				
Volumen [l]	100							
Protokoll-Nr. der nächsten Schritte	02/06	__/__	__/__	__/__	__/__	__/__	__/__	__/__

Entsorgung:

Sterilisation Abluftfilter	Datum: Zeit: Dauer:
Inaktivierung Fermenter	gesamter Inhalt o restl. Inhalt o Vol:____ [l] Überstand o
	Datum: Uhrzeit: Dauer: Temp.:
Besonderheiten	
Betriebs-Ende	Datum: [redacted] Uhrzeit: 1500

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Verlaufs-Protokoll: Ferm.Nr.: _____ Kostenstelle: _____ Vers.Nr.: 96/ _____ /02/ __

Organismus: _____

Betreiber: _____

Datum	Zeit	Probe Vol.						Zugabe	Para-meter	Wert alt	Wert neu	Bemerkungen
[redacted]	8.10	200ml	NBL	ik1								
[redacted]	7.40	150ml	Gel G									
[redacted]	8.25	200ml	NBL									

Fermentations- und Aufarbeitungsprotokoll

4-27

Technikumsleitung	:	Dr. A. Roß	Tel. 130	priv.: (0 53 1)	34 35 49
Fermentation	:	H. Schüler	Tel. 131	priv.: (0 53 71)	79 48
Aufarbeitung	:	R. Krützfeldt	Tel. 137	priv.: (0 53 02)	45 62
Technik	:	10 53 10	Tel. -	priv.: 0	

Auftragsnummer: [redacted] Reaktor-Nr.: [redacted] Anmeldedatum: 00.00.00

Name: Fischer

Berichtsempfänger: NBI

Dienstteldon: 465

Privatteldon:

Stamm/Medium: Soce 90

o Stammanmeldung liegt vor / X liegt bei

Ziel: Produktion von Epothilon

Prozeßbeginn am: [redacted] um 10[45] Uhr

Prozeßende am ca.: [redacted] um 10[00] Uhr

Startvolumen: 750 l

Volumen der Vorkultur + zusätzl. Zugaben: 70 l

Vorkultur: Schüttelkultur o Anfordarer o Biotechnikum o Reaktor/Exp.Nr.

Medium: Nr. E X trocken o gelöst ca. kg / l X AS

o wird vom Biotechnikum angesetzt o wird geliefert von am

Vorlage	Art	Anzahl	/ Konz.	/ Menge (l oder kg)	Pumpentyp	Rate (max.)
1	Lauge	KOH	10 %			
2	A.S.	Tegosipon				
3						
4						
5						

Waagen o 1 o 2 o 3 o 4 o 5 Timer o 1 o 2 o 3 o 4 o 5

o pH-Einstellung vor Sterilisation auf 7,6 mit ca. ml/l KOH

Sterilisation bei 121°C für min / fraktioniert h / Sterilluft h

Startwerte für die Kultivierung

Temperatur:	30 °C	Belüftung:	0,1 vvm Nm³/h
Drehzahl:	200 rpm	pH-Wert:	> 7,0 konstant
Druck:	mbar		

pO2-Messung	o nein	X ja		
Abgasmessung	o nein	o ja, Kanal		GVO o ja
pO2-Regelung	o nein	o ja, Sollwert	% Sättigung	
Druckregelung	o nein	o ja, Sollwert	mbar	
Reaktorerfassung	o nein	o ja, Exp.Nr.		

Parameter ändern

nach h --> nach h -->

nach h --> nach h -->

nach h --> nach h -->

weitere Angaben siehe umseitig ------>

Das Fermentationsprotokoll sollte in der der Fermentation vorangehenden Kalenderwoche (möglichst vorliegen, spätestens jedoch zwei Tage vor Beginn der Fermentation. Mündliche Fermentationsreservierungen werden nur bis zu diesem Zeitpunkt berücksichtigt.
Der Nutzer verpflichtet sich, nicht abgesprochene Manipulationen an Geräten zu unterlassen und die Technikum die Sicherheitsvorschriften (z.B. UVV 102) einzuhalten.

4-28

Anmerkungen/Besonderheiten zur Fermentation: Medium mit Ultraturrax suspendiert, 200 ml Tegospon, XAD-Zugabe

Aufarbeitung

Zielsetzung: Nach Rücksprache mit Hr. Steinmetz

Feststoffabtrennung:

o Zentrifugation o Mikrofiltration o Dead-end-Filtration

o Adsorberharzabtrennung

benötigt werden ---> o Filtrat/Überstand o Feststoff

o Lyophilisation o Ultrafiltration

Verdampfung gewünschtes Endvolumen :.....(L/ml)

max.Temp. :.....(°C)

Extraktion:

o Kulturbrühe o Überstand o Feststoff

Verteilungskoeffizient:.....

Lösungsmittel/Zusätze:.....

Phasenverhältnis:..... Stufenzahl:.....

Zusatzprotokolle:.....

Produktspezifische Besonderheiten/weitergehende Aufarbeitungsschritte/Analytik:

Toxische Eigenschaften/Sicherheitsmaßnahmen:.....

Besonderheiten der Entsorgung/Dekontamination von Mikroorganismen bzw. toxischen Produkten:

ACHTUNG!! Lagerzeiten von Kühlgut max.3 Arbeitstagen, von Gefriergut max.3 Monate!! Nach Terminüberschreitung erfolgt Entsorgung!!)

Datum/Unterschrift: [redacted] CFiSch

Wägeprotokoll F 900

ersuchsnummer: [redacted]

likroorganismus: Soce 40

ode für Medium: E - Medium

ehälterbeschriftung:

1	
2	
3	
4	

atum: [redacted]

nterschrift: C. Fiss

Nr.	Substanz	Firma	Nr. / batch	Soll	Einwaage	kg / g / mg / l
1	Magermilch			3kg	3,0	kg
2	Yeast extrakt	Ciba		1,5kg	1,5	kg
3	Stärke			7,5kg	7,5	kg
4	$CaCl_2$			750g	750,02	g
5	$MgSO_4$			750g	750,02	g
6	Fe-EDTA			6g	6,03	g
7	Soyamehl			3kg		kg
[redacted]	Glycerin	Roth		3,7l	3,7	l

nweise: Die Versuchsnummer wird bei Abgabe des Anmeldeprotokolls vergeben / Mediumscode und Bezeichnung des Mikroorganismus wie im Anmeldeprotokoll / das Protokoll kann für mehrere Behälter benutzt werden, die Nr. in der ersten Spalte ist die Behälternummer (siehe 'Behälterbeschriftung') / bei Lösungen unter Substanz z.B. Wasser aufführen und Menge in ml oder l angeben / werden Stammlösungen verwendet, ist deren Zusammensetzung beizufügen

FERMENTER: Blatt 1 Kostenstelle: 203310 Vers.Nr.: 96/ 155 /02/06

Betreiber: K. Seitz **Betreuer:** Sterlenski

Organismus: Suc 90

Kulturführung: Aerob: o Anaerob: o Phototroph: o

Prozeßführung: Batch: o Feed-Batch: o Konti: o

Fermenteraufbau:

Fermenter Nr. 2m0 1	Verwendung: Fermentation d Vorlage o	Steriltest o für Protokoll-Nr. __/__
Sicherheitsmaßnahmen	Abluftfilter: Nein o Ja o Handschuhe tragen: Nein o Ja ~~o~~	
Betrieb-Beginn	Datum: [redacted]	Uhrzeit: 10⁰⁰
Rührerart	2x Scheib	
Sondergeräte		
Pumpe für Lauge	Typ:	Pumprate: Durchm$_{Schlauch}$:
Pumpe für	Typ:	Pumprate: Durchm$_{Schlauch}$:
	Medium mit Hefeheber	

Elektroden:

pH-Elektrode	Nr.: 200.6	Puffer 1: 7 Poti/ ~~mV~~: -5,1	Puffer 2: 4 Poti/ ~~mV~~: 55,7
pH-Elektrode	Nr.:	Puffer 1: Poti/ mV:	Puffer 2: Poti/ mV:
pO_2-Elektrode	Nr.: 150.16	Nr.: 150.15	

Reaktorgewicht: Gesamtgewicht

Sollgewicht	680	[KG]
leer		[KG]
Wassermenge	580 [l]	[KG]
Medium-Zugabe	Name: Herk.: Nutzer 0 SE: 03/04	650 [KG]
	XAD Zugabe: ~~o~~ Ja o Nein	
Antischaum	Art: Origin Volumen: 2m0 [ml]	
pH vor Sterilisation	Ist: 6,18 Soll: 7,0	
pH eingestellt mit	Name: KOH Konz.: 5N Menge: 360 ml	

Sterilisation:

Steril. Gleitringdichtung	Datum: [redacted]	Uhrzeit: 9⁴⁰	Dauer: 50	min	
1. Sterilisation Fermenter	Datum: [redacted]	Uhrzeit: 11¹⁰	Dauer: 60	min	
2. Sterilisation Fermenter	Datum:	Uhrzeit:	Dauer:	min	
pH nach Sterilisation	6,34	Reaktorgewicht nach St.		kg	

Substrat und Hilfsmittel Zugabe:(nach Sterilisation)

Art	Herkunft	Vol. [ml]	Datum	Zeit	Gew.$_{gesamt}$(KG)
AS	Flasche Nr. 96/0537	300	[redacted]	7105	
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				
	Flasche Nr. ____				

Vorlagen und Korrekturmittel:

Lauge 1: KOH 10%	Vol.$_{Anfang}$: 4700	Dat./Zeit: [redacted] 1345	Herk.: Flasche Nr. 539
Lauge 2:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Lauge 3:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 1:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 2:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Säure 3:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Antischaum 1: Tegosipon	Vol.$_{Anfang}$: 1500	Dat./Zeit:	Herk.: Flasche Nr. 537
Antischaum 2:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 1 Art:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____
Zufütterung 2 Art:	Vol.$_{Anfang}$:	Dat./Zeit:	Herk.: Flasche Nr. ____

Regelung und Fermentationsstrategie, Startwerte:

pH-Sollwert:	eingestellt mit :		pH-Regelung von ..... bis 7,0
pO_2	Messung nein o	ja ø	Regelung nein ø o ja o
pO_2 Sollwert: 30%	Strategie: Drehzahl ø	Zuluft o	sonstige o ____
Temperatur 30 [°C]	Druck 300 [mbar]		Drehzahl 100 [rpm]
Parameter:	Sollwert: [.....]		Strategie:
Parameter:	Sollwert: [.....]		Strategie:
Parameter:	Sollwert: [.....]		Strategie:
Begasung: Luft 70 l/min			0,1? vvm
andere: ____	____ l/min		____ vvm
____	____ l/min		____ vvm
____	____ l/min		____ vvm
Überlagerung GLRD	Luft ø	Dampf o	
Abgasmessung	nein o	ja ø	Kanal: 2
Rechnererfassung nein o	Exp.: A61456	Start-Datum: [redacted]	Zeit: 1310

Fermenter: Blatt 3 Kostenstelle: 102320 Vers.Nr.: 96/145/02/06

Inokulation:

Inokulum 1	Herk.: Nutzer 0 Protokoll-Nr.: 02/03 Flasche Nr. ____	Volumen: 100 [l]
Fermentation Beginn	Datum: [redacted] Uhrzeit: 1310	
Inokulum 2	Herk.: Nutzer 0 Protokoll-Nr.: __/__ Flasche Nr. ____	Volumen: [l]
Inokulum 2 Zeitpunkt	Datum: Uhrzeit:	
Fermentergewicht nach	Inokulierung 1: 7780 [kG]	Inokulierung 2: [kG]

Fermentation-Ende:

Fermentation-Ende	Datum: [redacted] Uhrzeit: 0805		
Fermenter-Gewicht			
Korrekturmittel: Volumen nach der Fermentation	Säure 1:	Säure 2:	Säure 3:
	Lauge 1: 3000	Lauge 2:	Lauge 3:
	Antischaum 1: 1500	Antischaum 2:	
Volumen nach Ferm. von	Zufütterung 1:	Zufütterung 2:	
wie geplant x			
Kontamination o	Zeitpunkt: Vor den Animpfen o	Vorkultur o	
	Nach dem Animpfen o		
Defekt o	Art:		
übergeschäumt o	Zeitpunkt: Sterilisation:	Kultivierung:	
	Aufheizphase o	Vor den Animpfen o	
	Haltephase o	während der Kultivierung o	
	Abkühlphase o	am Ende der Kultivierung o	
sonstiges			

Weiterverarbeitung:

Transferleitung Sterilisat.	Datum: [redacted] Uhrzeit: 1035 Dauer:							
Ablassleitung Sterilisat.	Datum: Uhrzeit: Dauer:							
Nächster Schritt der Weiterverarbeiung:	Aufarbeitung x			Übergeimpft auf einen Fermenter 0				
	An Nutzer übergeben 0			Übergeimpft auf mehrere Fermenter 0				
Volumen [l]	776							
Protokoll-Nr. der nächsten Schritte	03/__	__/__	__/__	__/__	__/__	__/__	__/__	__/__

Entsorgung:

Sterilisation Abluftfilter	Datum: Zeit: Dauer:	
Inaktivierung Fermenter	gesamter Inhalt o restl. Inhalt o Vol:____ [l]	Überstand o
	Datum: Uhrzeit: Dauer: Temp.:	
Besonderheiten		
Betriebs-Ende	Datum: [redacted] Uhrzeit: 1502	

Verlaufs-Protokoll: Ferm.Nr.: _ _ _ _ . _ Kostenstelle: _ _ _ _ _ _ Vers.Nr.: 96/ _ _ _ _ /02/ _ _

Organismus:______

Betreiber:______

Datum	Zeit	Probe Vol.						Zugabe	Para-meter	Wert alt	Wert neu	Bemerkungen
[redacted]	8 20	200 ml	UBC									
[redacted]	15 35								Druck	500 mbar	300 mbar	Druck auf 300 mbar = 0,1 bar eingestellt
[redacted]	7 40	200 ml	Opt.d									Null liegt bei 300 mbar Überdruck
"	"								Druck	200 mbar	0 mbar	
[redacted]	7 50	200 ml	UBC									
[redacted]	8 50											pO2 Regelung ein 30%
												Min 100 upm max 300 upm
[redacted]	10 00											pO2 Regelung aus
[redacted]	7 45	200 ml	Opt.d									hat Zuluft [illegible] gefahren
[redacted]	8 40	200 ml	UBC									
[redacted]	14 30							AS				ca. 20 ml
[redacted]	17:30	~~500~~ ml						AS				300 ml Fermenter eingefüllt
[redacted]	12:35	400 ml		L								
[redacted]	7 30	100 ml										
[redacted]	8 00	100										
[redacted]	14 00	300										

[redacted] 8 00 300

[redacted] 8 10 900

[redacted]

Stammkultform

Soce 90 A4 atril

Soce 90 A3 atril

Soce 90 A2 atril

Soce 90wild atril

Soce 1275 atril

Soce 1798 AS atril

Soce 1300 atril

Beziehung

Begleitpersonsuche

Klon 7-38 Soce 90 atril

[redacted]

P76 Erbeutet

Probe F900 7700

Klon 7-34

970

P78 Ernte V

800 F900 Probe V

Aufbewahrung

-11°C

Dead-End-Filtration Kostenstelle: 103310 Vers.Nr.: 96/015E/03/06

Bearbeiter: Prozeß: Roth Analysen: Analysen-Protokoll: __/__

Stamm / Medium: F96 m Girth MRT

Besondere Sicherheitsmaßnahmen: Handschuh

Zielsetzung: XAD Gewinn

Grunddaten

Anlagentyp	Prozeßfilter Wiga EFT 60/180
Modifikationen	Process filter
Betriebsbeginn	Datum: [redacted] Uhrzeit: 08:[redacted]
Prozeßbeginn	Datum: [redacted] Uhrzeit: 08:20

Bearbeitetes Material

Art: Fermentationsbrühe		
Vol.: 750 [l]	Temp. [°C]	Herk.: Nutzer o Protokoll-Nr: 02/06
pH [-]	oD_{Medium} [-]	Feststoffanteil [g/l]

Filterhilfsmittel

Art:	eingewogen [kg]	gelöst in [l]
$Konz_{Filterhilfsmittel}$ [g/l]	konti.Dosierung [l/h]	Pumpentyp:

Geräteparameter

Eingesetzte Filtermedien/Siebgewebe: 250 M.

Produkte

Gesamtlaufzeit 5,5 [h]	mittlerer Flux 0,126 [l/h]	$oD_{Klarlauf}$ [-]
$Feststoff_{Klarlauf}$ [mg/l]	$Endvol._{Klarlauf}$ [l]	$Prodkonz_{Klarlauf}$ [.../ml]
$Feststoff_{Konzentrat}$ [mg/l]	$Endvol._{Konzentrat}$ [kg]/[l]	$ProdKonz_{Konzentrat}$ [.../ml]
Konz.grad [%]	Konz.faktor [-]	

Verbleib der Produkte

	Klarlauf	Konzentrat XAD
Weiterverarbeitung	Protokoll-Nr /	Protokoll-Nr 03/
An/Für Nutzer	übergeben o eingelagert o	übergeben o eingelagert o
Inaktivierung (Art)		
Entsorgung (Art)	X	

Versuchsende

Prozeßende	Datum [redacted] Uhrzeit: [illegible]
Betriebsende	Datum [redacted] Uhrzeit: [illegible]

Versuchsprotokoll:

Probe Nr	Datum Uhrzeit	Laufzeit [min.]	Differenz-druck x10³[hPa]	Flux [l/h]	Rückspül-zyklen [min.]			

Bemerkungen

Feststoffextraktion / Desorption Kostenstelle: 103116 Vers.Nr.: 96/0151/03/07

Bearbeiter: Prozeß: Roth Analysen: Analysen-Protokoll: __/__

Stamm / Medium: E. coli Gerth Mix T Ampicillin

Besondere Sicherheitsmaßnahmen:

Zielsetzung:

Grunddaten

Anlagentyp	Seitz Pressfilter EFT 60/180
Modifikationen	
Betriebsbeginn	Datum: [redacted] Uhrzeit: 14:30
Prozeßbeginn	Datum: [redacted] Uhrzeit: 15:00

Bearbeitetes Material

Art:		12 g Produkt erwartet	
Vol.: [l]	Menge: [g]	Herk.: Nutzer o	Protokoll-Nr: /

Extraktions-/ Desorptionsverlauf

Probe Nr.	Menge [kg] ; [l]	Lösungsmittel Art	Menge[l]	Kontaktzeit [min] ; [h]	Produkt [mg] ; [g]	Phasentrennung Q_m [l/h]
Reinigung	ca. 15	Methanol 1.)	45	über Nacht		
1 Ansatz	15 Kg	Methanol	15	3 h		
2 Ansatz	15 Kg	Methanol	15	über das Wochenende		
3. Ansatz	15 Kg	Methanol	15	3 h		
4 Ansatz	15 Kg	Methanol	30 L	3 h		
5 Ansatz	15 Kg	Methanol	30 L	ü. Nacht		
6 Ansatz	15 Kg	Methanol	30 L	3 h		
7.	15 Kg	Methanol	15 L	2 h		

1.) Methanol + H_2O Gemisch 12 + 2 !

Verbleib der Produkte:

	Wässrige Phase	Organische Phase
Weiterverarbeitung	Protokoll-Nr.: /	Protokoll-Nr.: 03/09
An/Für Nutzer	übergeben o eingelagert o	übergeben o eingelagert o
Inaktivierung (Art)		
Entsorgung (Art)	⊠	

Versuchsende

Prozeßende	Datum: [redacted] Uhrzeit: 17 : 00
Betriebsende	Datum: [redacted] Uhrzeit: 17 : 30

Bemerkungen:

Verdampfung Kostenstelle: 1033.10 Vers.Nr.: 96/0195/03/08

Bearbeiter: Prozeß: Petrick Analysen: Analysen-Protokoll: __/__

Stamm / Medium: [illegible]

Besondere Sicherheitsmaßnahmen: Handschuhe

Zielsetzung: Aufkonzentrierung der Methanolphase

Grunddaten

Verdampfertyp	Luwa Dünnschichtverdampfer	
Modifikationen Besonderheiten	thin film evaporator	
bei Dünnschicht-verdampfer	Starrflügelrotor X	Schwingflügelrotor 0
Betriebsbeginn	Datum: [redacted]	Uhrzeit: 09:30
Prozeßbeginn	Datum: [redacted]	Uhrzeit: 09:45

Bearbeitetes Material

Art: Methanol (Luwa)			
Vol.: 150 + [l]	Konz$_{Rohprodukt}$ [g/l]	Herk.: Nutzer o	Protokoll-Nr: /

Rotationsverdampfer

Vakuum 140 [hPa]	Brüdentemperatur [°C]
Badtemperatur [°C]	

Dünnschichtverdampfer

Vakuum 140 (120) [hPa]	
Rohproduktflux [l/h]	Rohproduktpumpe 46 (48) [%]
Destillatleistung 53 [l/h]	Konzentratflux [l/h]
Eindampfverhältnis [-]	Dampfvordruck 0,5 Bar abs. (2) x10³[hPa]
Konzentrattemperatur 30,5 [°C]	Brüdentemperatur 23 [°C]
Wärmeträger Zufuhr 100 [°C]	Wärmetr. Abfuhr 78 [°C]

Rektifikation

Vakuum [hPa]	
$T_{Destillat}$ [sec.]	$T_{Rücklauf}$ [sec.]
Destillatleistung [l/h]	Dampfvordruck x10³[hPa]
Destillatmenge [l]	Sumpfprodukt [l]

(* Schaumprobe stieg stetig an, Vakuum herangesetzt).

(2) Dampfvordruck auf 0,4 Bar gesenkt.

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Batch-Reaktor

Vakuum		Konzentrattemp. [°C]	
Destillatleistung		Heizmedium [°C]	
Direkt-bedampfung	Dampfvordruck	x10³[hPa]	
	Destillatleistung [l/h]	Konzentrattemp. [°C]	

Produkte

End-Werte	Endvolumen [l]	$Konz_{Produkt}$ [ ]	Bemerkungen
Konzentrat	26 L		
Sumpf	130 L		

Ausbeute: Produktmenge Konzentrat/Menge Rohprodukt x 100 = [%]

Verbleib der Produkte

	Konzentrat	Sumpf Destillat
Weiterverarbeitung	Protokoll-Nr.: 031	Protokoll-Nr.: /
An/Für Nutzer	übergeben o eingelagert o	übergeben o eingelagert ø
Inaktivierung (Art)		entsorgt Destillat
Entsorgung (Art)		

Versuchsende

Prozeßende	Datum: [redacted] Uhrzeit 15:00
Betriebsende	Datum: [redacted] Uhrzeit 16:00

Bemerkungen: Konzentrat Dünnschichtverdampfer in Rotiverdampfer überführt, um Rotiverdampfer mit Methanol abzudrücken.
Wasserbadtemp. 28°C, Max. Unterdruck 51 m.Bar.

Flüssig-(Fest-)Flüssig - Extraktion Kostenstelle: 103310 Vers.Nr.:96/0195/03/09

Bearbeiter: Prozeß: [illegible] Analysen:............ Analysen-Protokoll: __/__

Stamm / Medium: Konzentrat [illegible] [illegible] [illegible] Dünnschichtverdampfer

Besondere Sicherheitsmaßnahmen:............

Zielsetzung: Gegenextraktion zur Wasserphase

Grunddaten

Extraktortyp	[illegible] QVF / SA1 Separator	
Modifikationen		
Betriebsbeginn	Datum: [redacted]	Uhrzeit: 14:3.
Prozeßbeginn	Datum: [redacted]	Uhrzeit: 15:..

Bearbeitetes Material

Art: Konzentrat Dünnschichtverdampfer - Rotationsverdampfer		
Vol.: 26 [l]	Herkunft: Nutzer o	Protokoll-Nr.: /
Temp.: [°C]	pH [-]	Konz.$_{Produkt}$ [mg /....]

Zusätze

Zusatzstoffe	Art: Ammoniak 1 zu 2 PH >
pH-Einstellungen	mit: 1 zu 4 Essigsäure [illegible] lauf: 6,95
Lösungsmittel	Art: Ethylacetat

Betriebsdaten

Durchfluß Phase$_{Wässrige}$	[l/h]	Durchfluß Phase$_{Organ}$	[l/h]	Phasen-verhältnis	1 zu 1 [-]

Extraktionsverlauf

Probe Nr.	Datum Uhrzeit	Stufe	Konz. [mg/l] Phase$_{Wässrige}$	Phase$_{Organ}$	Vol. [l] Phase$_{Wässrige}$	Phase$_{Organ}$	Bemer-kungen
1 Extrakt		1.			20	20	sehr [illegible]
2 Extr.		2.					
		3.					
Start	5 4 2	4.			ca. 30% Emulsion		
		5.					
		6.					

Phasentrennung

Dekantierung o	Filtration (Koaleszenz) o	Zentrifugation X

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Volumen der Produkte

$Phase_{Wässrige}$	SA1 Endvol.: 43 [l]	$Phase_{Organisch}$	Endvol SA1 46 L [l]

Konditionierung der Produkte

Phasen	Zusatzstoffe (z.B. Na_2SO_4) Art	Menge [g];[l]	pH-Einstellung mit	auf
Wässrige				
Organische				

Verbleib der Produkte:

	Wässrige Phase	Organische Phase
Weiterverarbeitung	Protokoll-Nr.: /	Protokoll-Nr.: 03 / 20
An/Für Nutzer	übergeben o eingelagert o	übergeben o eingelagert o
Inaktivierung (Art)		
Entsorgung (Art)	x	

Versuchsende

Prozeßende	Datum: [redacted]	Uhrzeit: 13:45
Betriebsende	Datum: [redacted]	Uhrzeit: 15:30

Bemerkungen: Auf Grund ungünstigen Trennungsverhältnis wurden weitere 25 L Ethylacetat + 25 L H_2O hinzugefügt (16.10.20). 88 L Gesamtvol.
Dekantierung nicht erfolgreich.
Emulsion PU ingestalt, Trennung über Separator SA1.

Verdampfung

Kostenstelle: 103110 Vers.Nr.: 96/0195/03/10

Bearbeiter: Prozeß: Pirsith Analysen: .... Analysen-Protokoll: __/__

Stamm / Medium: Ethylacetat Extrakt F.Gaa.: Gitrho Lipukyl

Besondere Sicherheitsmaßnahmen: ....

Zielsetzung: Konzentrierung zum Produkt

Grunddaten

Verdampfertyp	Rotationsverdampfer QVF R20DEX	
Modifikationen Besonderheiten		
bei Dünnschicht-verdampfer	Starrflügelrotor 0	Schwingflügelrotor 0
Betriebsbeginn	Datum: [redacted]	Uhrzeit: 07:15
Prozeßbeginn	Datum: [redacted]	Uhrzeit: 07:50

Bearbeitetes Material

Art: Ethylacetat-Extrakt			
Vol.: 46 [l]	$Konz_{Rohprodukt}$ [g/l]	Herk.:Nutzer o	Protokoll-Nr: /

Rotationsverdampfer

Vakuum 96 [hPa]	Brüdentemperatur 21,2 [°C]
Badtemperatur 27,7 [°C]	

Dünnschichtverdampfer

Vakuum [hPa]	
Rohproduktflux [l/h]	Rohproduktpumpe [%]
Destillatleistung [l/h]	Konzentratflux [l/h]
Eindampfverhältnis [-]	Dampfvordruck x10^3[hPa]
Konzentrattemperatur [°C]	Brüdentemperatur [°C]
Wärmeträger Zufuhr [°C]	Wärmetr., Abfuhr [°C]

Rektifikation

Vakuum [hPa]	
$T_{Destillat}$ [sec.]	$T_{Rücklauf}$ [sec.]
Destillatleistung [l/h]	Dampfvordruck x10^3[hPa]
Destillatmenge [l]	Sumpfprodukt [l]

Batch-Reaktor

Vakuum		Konzentrattemp. [°C]	
Destillatleistung		Heizmedium [°C]	
Direkt-bedampfung	Dampfvordruck	x10³[hPa]	
	Destillatleistung [l/h]	Konzentrattemp. [°C]	

Produkte

End-Werte	Endvolumen [l]	$Konz_{Produkt}$ [ ]	Bemerkungen
Konzentrat	ca. 4 L	ca. 4 L	
Sumpf	42 L		

Ausbeute: Produktmenge Konzentrat/Menge Rohprodukt x 100 = [%]

Verbleib der Produkte

	Konzentrat	Sumpf
Weiterverarbeitung	Protokoll-Nr.: /	Protokoll-Nr.: /
An/Für Nutzer	übergeben x eingelagert o	übergeben o eingelagert x
Inaktivierung (Art)		
Entsorgung (Art)		

Versuchsende

Prozeßende	Datum: [redacted] Uhrzeit: 01:36
Betriebsende	Datum: [redacted] Uhrzeit: 11:00

Bemerkungen:

EPOTHILON - Aufarbeitung 900l (750l AV)

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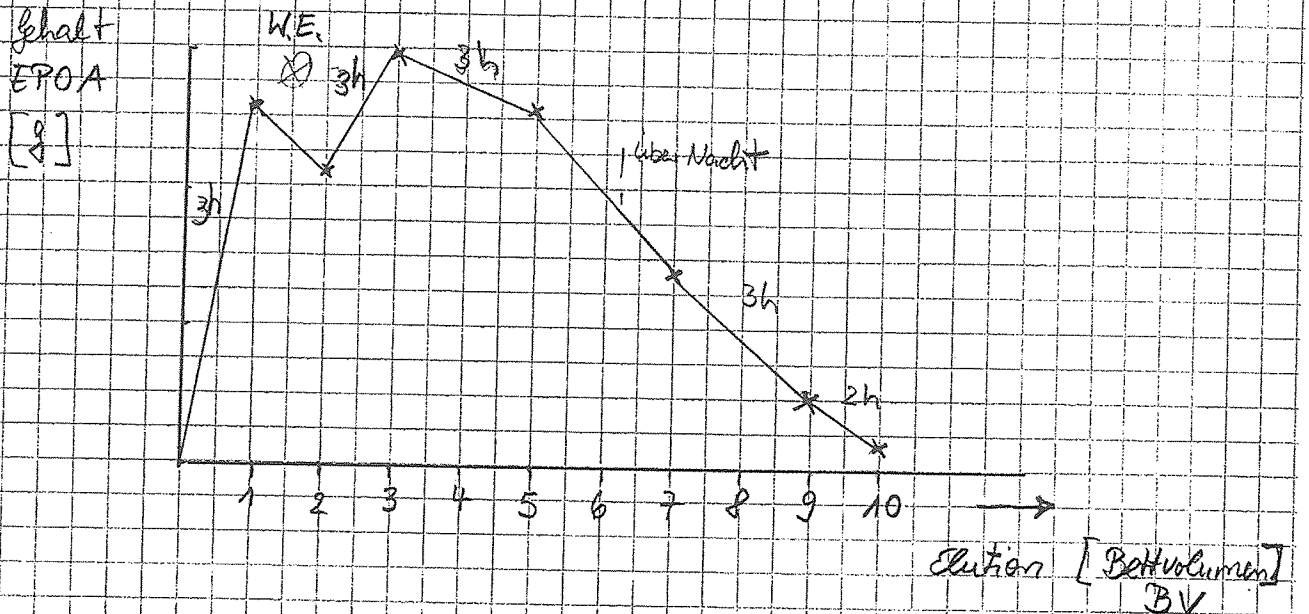
XAD: ca 15l

Analytik Integral 3500 ≙ 4,1 µg EPOB

XAD-Elution

			EPOTHILON AB-Gehalt	(g)
1.	MeOH/Wasser	1:2 (ca 30l)	—	—
2.	MeOH	15l	2,60	1,40
3.	"	15l	2,10	1,23
4	"	15l	2,95	1,80
5	"	30l	2,63	1,58
6	"	30l	1,37	0,80
7	"	30l	0,50	0,32
8	"	15l	0,22	0,14
			12,37	7,27

Gesamtelutionsvolumen: 150l ≙ 10 BV



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Method name: C:\HPCHEM\1\METHODS\SCREEN1.M
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Injection Time: 10:36:36 AM
Sequence Name:
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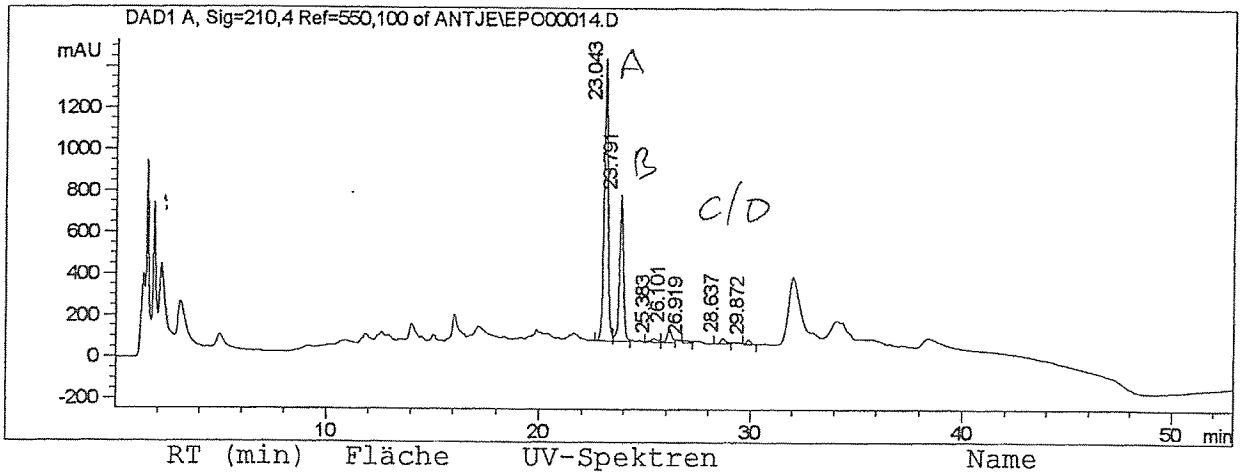

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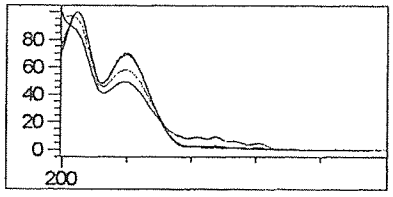
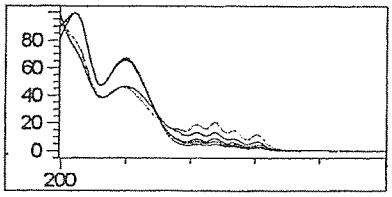
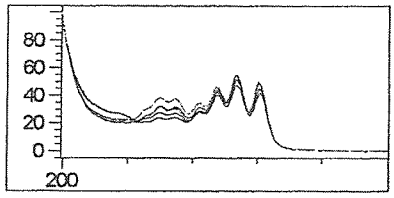
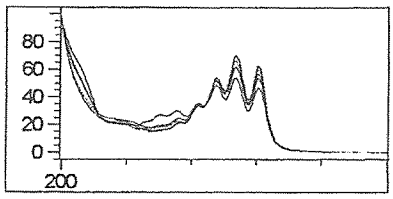
23.04	17886.2		
23.79	9602.7		
25.38	439.0		
26.10	1218.6		

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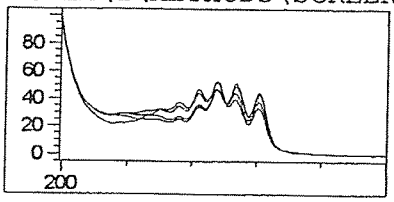
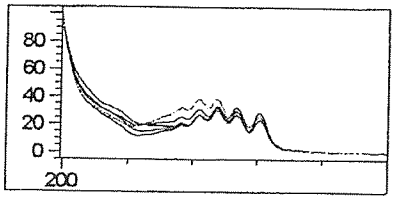
4-47

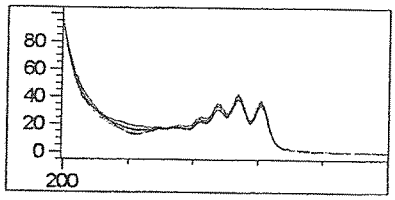
26.92 324.4



28.64 519.7



29.87 367.6



4-48

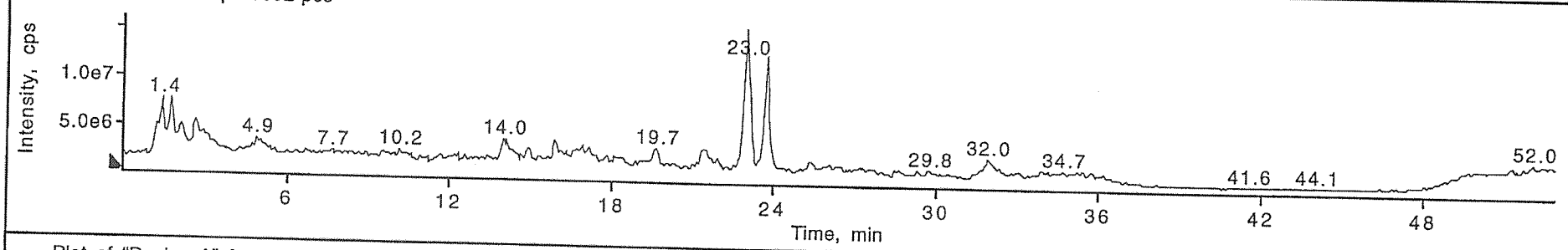
Integration Results

Signal 1: DAD1 A, Sig=210,4 Ref=550,100

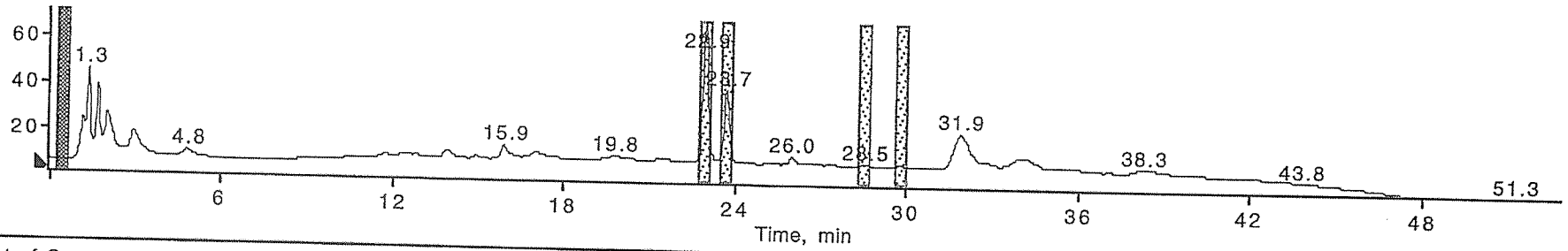
5µL

Peak#	Time [min]	Type	Area [mAU*s]	Height [mAU]	Width [min]	Start [min]	End [min]
Epo A 1	23.043	PV	17886.182	1359.741	0.201	22.614	23.457
Epo B 2	23.791	VV	9602.701	705.784	0.206	23.457	24.265
3	25.383	PV	438.981	17.453	0.335	24.965	25.738
4	26.101	VV	1218.557	71.913	0.252	25.738	26.433
5	26.919	VV	324.419	11.990	0.364	26.720	27.240
6	28.637	PV	519.701	26.924	0.279	28.245	29.056
7	29.872	VV	367.567	23.116	0.235	29.647	30.264

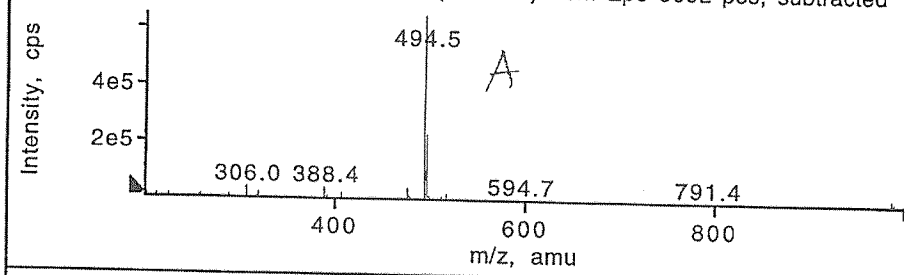
Plot of TIC from Epo 900L pos



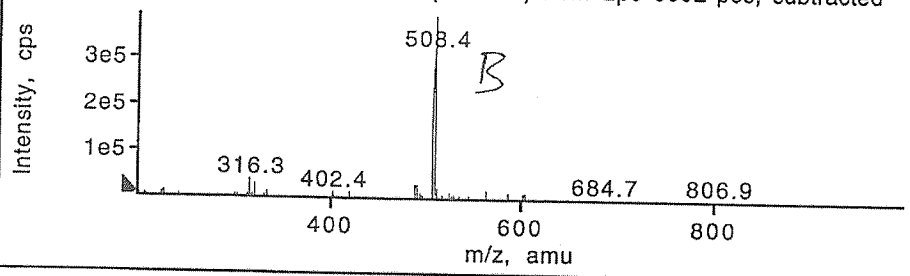
Plot of "Device A" from Epo 900L pos



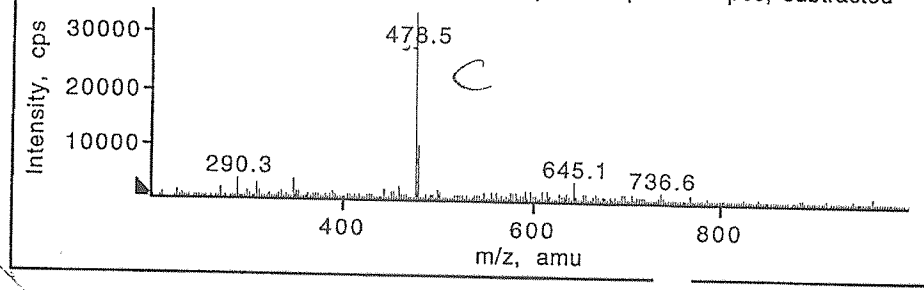
Plot of Spectrum from 22.95 min (7 scans) from Epo 900L pos, subtracted



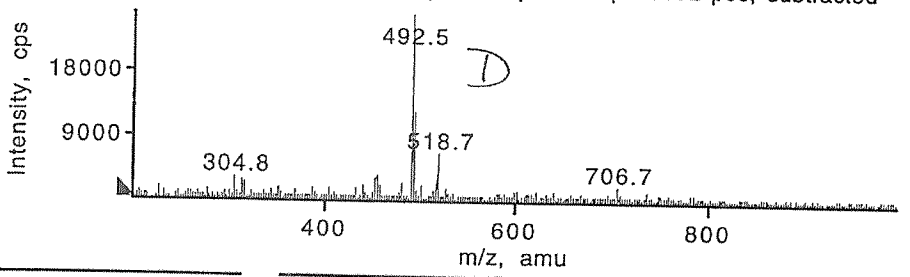
Plot of Spectrum from 23.71 min (8 scans) from Epo 900L pos, subtracted



Plot of Spectrum from 28.55 min (7 scans) from Epo 900L pos, subtracted



Plot of Spectrum from 29.82 min (7 scans) from Epo 900L pos, subtracted



XAD-Eluate 1-8 über Eindampfkolben konzentriert und ausschließend mit EE verteilt

Wasserphase 42 l — ca 280 mg EPO in H_2O-Phase
EE-Phase 46 l

- Wasserphase wurde nun 1 mal extrahiert
- EE-Phase wurde im Roti konzentriert und von NCH übernommen.
- EE-Extrakt war sauer pH 5,0 und wurde mit 1M NH_4COOCH_3 gepuffert. Schwierigkeiten bei der Pufferein stellg.
- Nachteil bei EE-Konzentrierung dampft NH_3-Gas ab. Das Eluat wird sauer.
- Erneute Pufferung mit 0,5 M KPO_4-Puffer
- Extraktion ist neutral!
- EE-gesamt-Extrakt 246 g 407.
  Gehalt EPO A = 13,82 g A
  " " B = 8,40 g B
- von den 246 g Rohextrakt wurden 80 g entnommen und einmal mit n-Heptan ausgeschüttelt!
- n-Heptanphase : 20 g
  MeOH-Phase : 60 g 408.

Essigsäure Extrakt .407 (2/3 davon)
(1/3 s. 408)

wurden mit Heptan - verteilt 413. Auswaage:

Heptanphase: 20.4 g

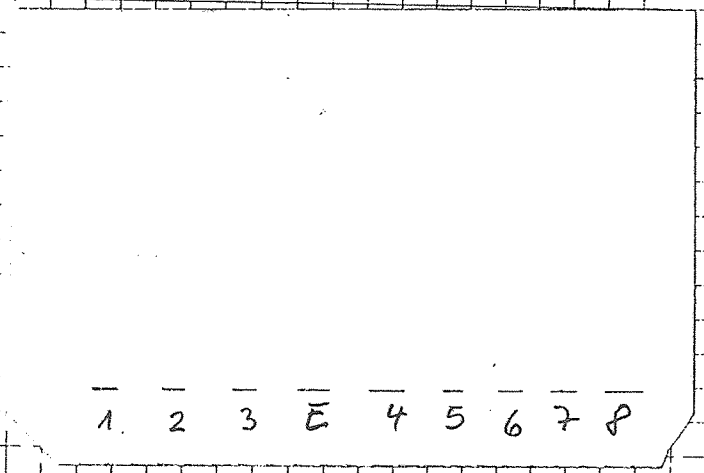
EPOA 30 mg verworfen

" B 19 mg "

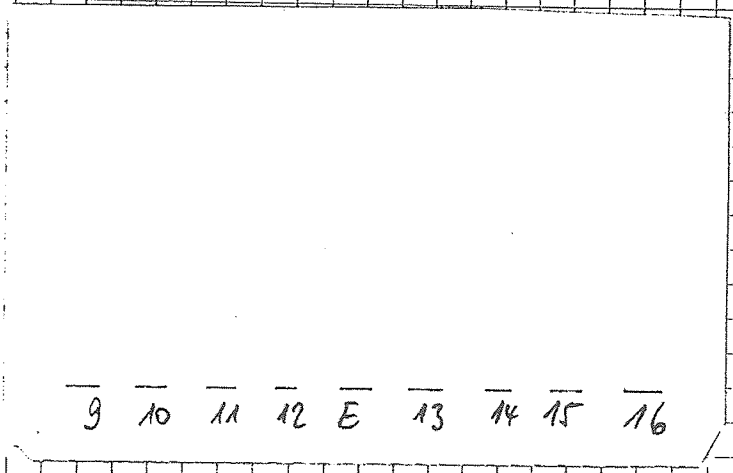
[redacted]

LH20 - Chromatographie von So 90. 408 900 l 4-52

Teil 1 60g Rohextrakt



↓ Spirangien
Spots for epo-
thilones faded



Fraktionierung 1 – 3 ~~409.~~ weg

4 + 5 Spirangiene 410.

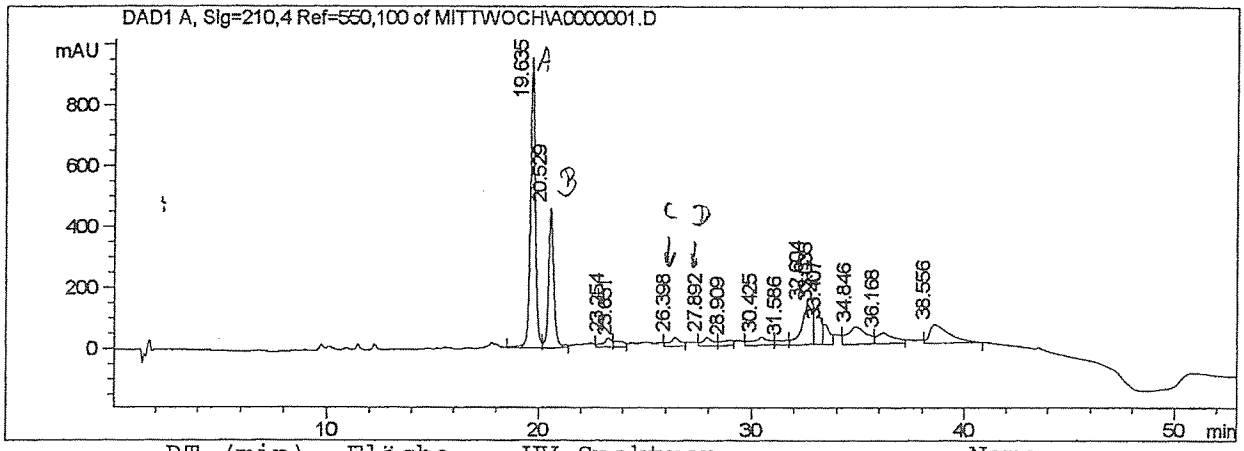
6 – 12 Spirang + EPO 411.

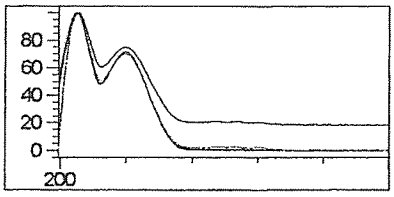
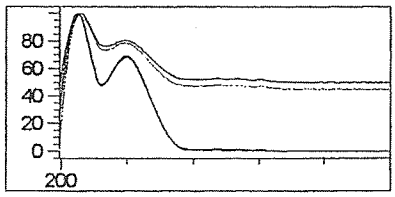
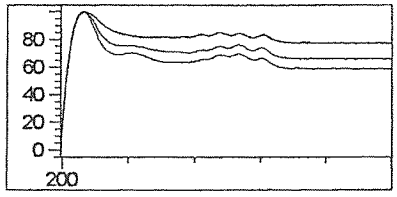
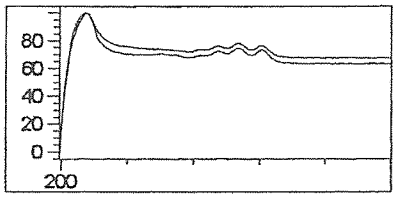
13 – Rest (19) 412.

Data File name: C:\HPCHEM\1\DATA\MITTWOCH\A000000->
Method name: C:\HPCHEM\1\METHODS\SCREEN1.M
Sample Name: Fr.6-12 Sample Info:
Injection Time: 4:02:31 PM
Sequence Name:
Report Style: screen1
data acquired by:Antje on: [redacted] 4:02:31 PM
vial:1

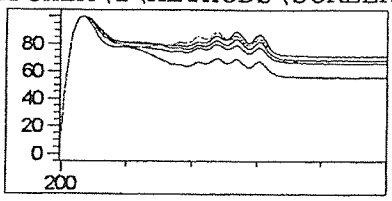
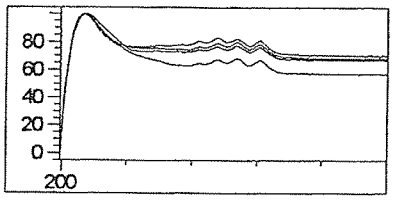
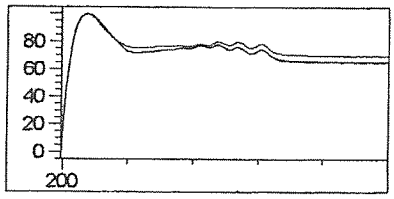
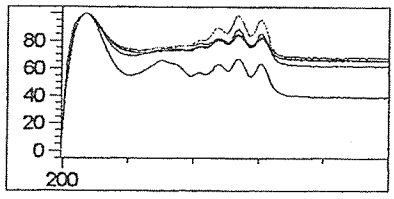
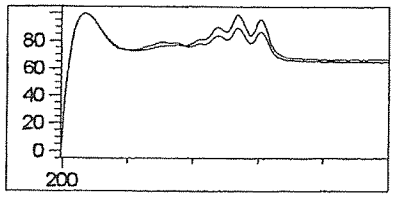
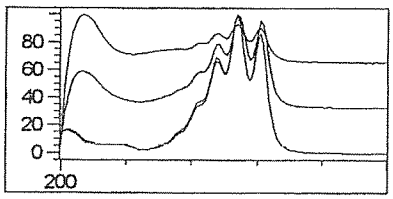
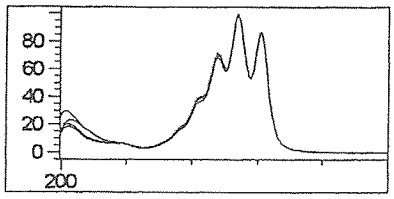
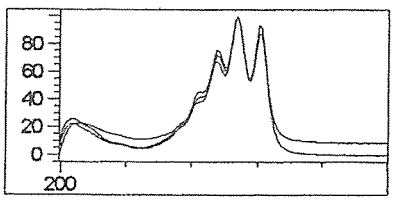
4-53

LH20



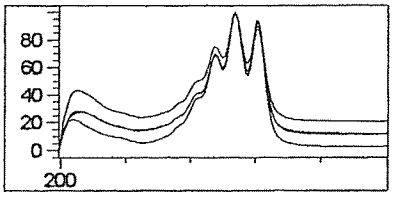
RT (min)	Fläche mAU*sec	UV-Spektren	Name
19.63	15304.8		
20.53	7617.5		
23.25	894.8		
23.65	648.1		

Y-54

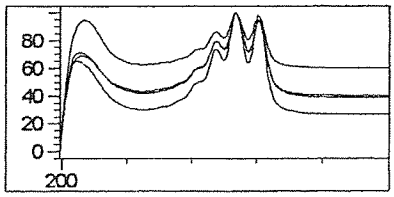
26.40	1092.9	
27.89	956.6	
28.91	706.5	
30.42	1476.6	
31.59	640.9	
32.60	4815.9	
33.04	2642.5	
33.41	1459.5	

4-55

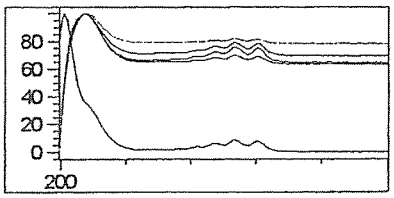
34.85 3627.0



36.17 2105.6



38.56 4021.5



Data File C:\HPCHEM\1\DATA\MITTWOCH\A0000001.D Sample Name: FI. 6-12

Fl. 6-12

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Integration Results

Signal 1: DAD1 A, Sig=210,4 Ref=550,100

Peak#	Time [min]	Type	Area [mAU*s]	Height [mAU]	Width [min]	Start [min]	End [min]	
1	19.635	VV	15304.782	955.700	0.241	18.503	20.154	A
2	20.529	VV	7617.517	457.590	0.249	20.154	21.403	B
3	22.436	VV	533.199	14.543	0.483	21.932	22.681	
4	23.254	VV	894.821	30.803	0.393	22.681	23.520	
5	23.651	VV	648.111	19.969	0.421	23.520	24.170	
6	26.398	VV	1092.884	30.130	0.485	25.898	26.916	C
7	27.892	VV	956.623	28.627	0.448	27.530	28.423	D
8	28.909	VV	706.516	16.989	0.574	28.423	29.178	
9	30.425	VV	1476.579	27.183	0.694	29.676	31.044	
10	31.586	VV	640.901	16.239	0.506	31.044	31.743	
11	32.604	VV	4815.902	130.967	0.544	31.743	32.884	
12	33.035	VV	2642.511	127.065	0.294	32.884	33.321	
13	33.407	VV	1459.489	68.039	0.298	33.321	33.810	
14	34.846	VV	3626.960	57.609	0.901	34.234	35.738	
15	36.168	VV	2105.642	36.447	0.792	35.738	37.210	
16	38.556	VV	4021.517	61.308	0.907	38.050	40.871	

6 ✓
1 ✓
400 mg ✓
400 mg ✓

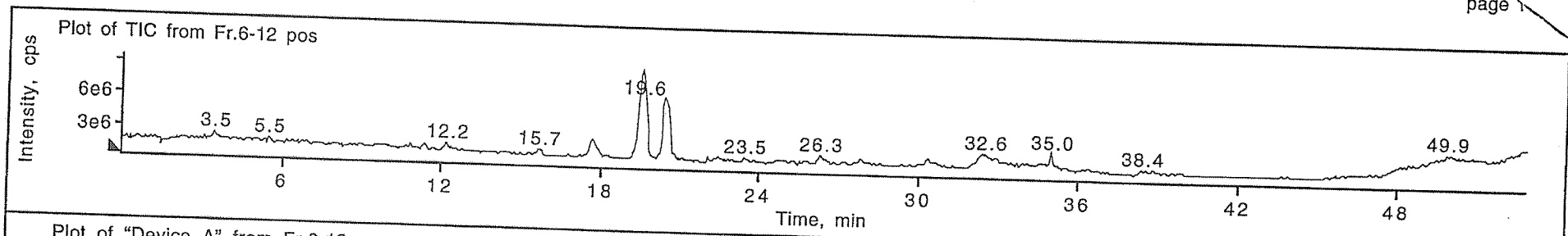
5800 – 2 µg
1000

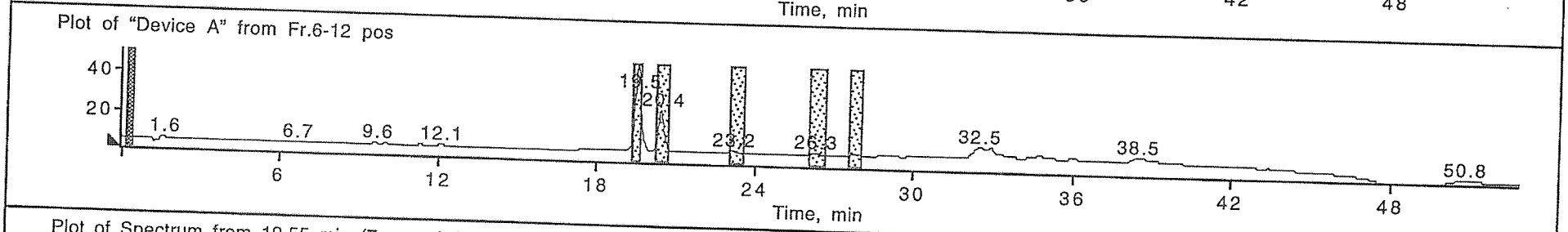
4,1 mg/ml
2,0 µg

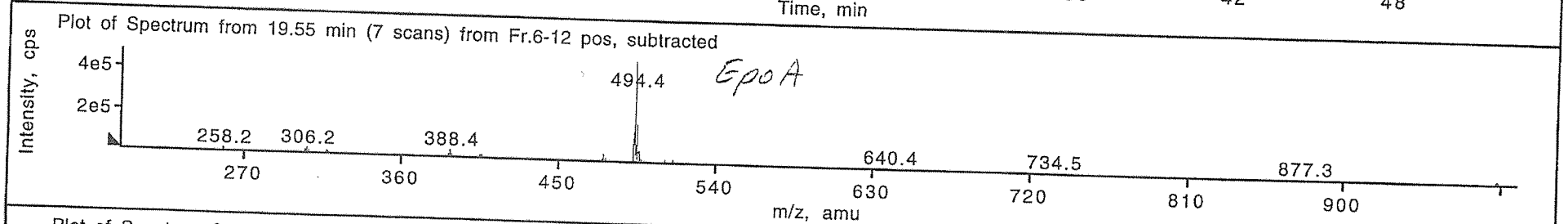
5 µl

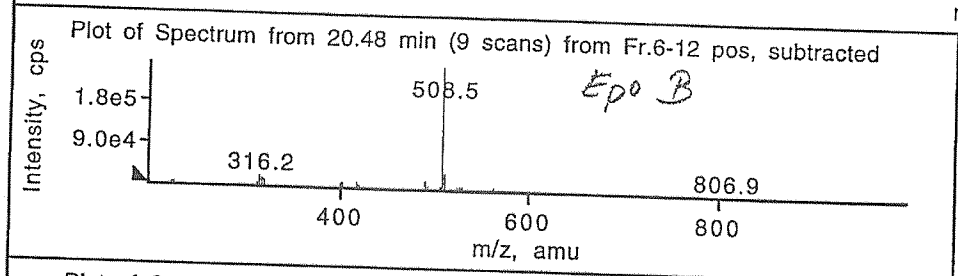
$$\frac{2000 \cdot 200 \cdot 5800}{5800 \cdot 1000} = 400 \text{ mg}$$

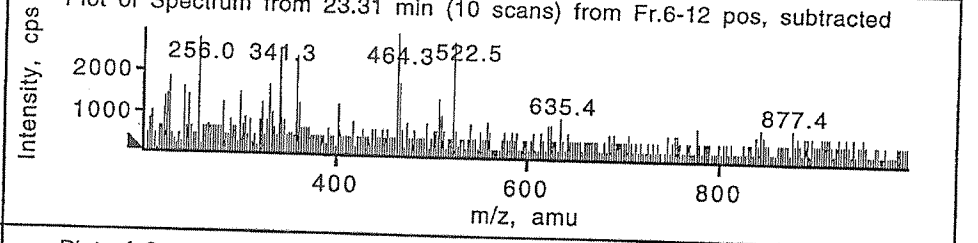
Instrument 1 [redacted] 8:16:13 AM Antje

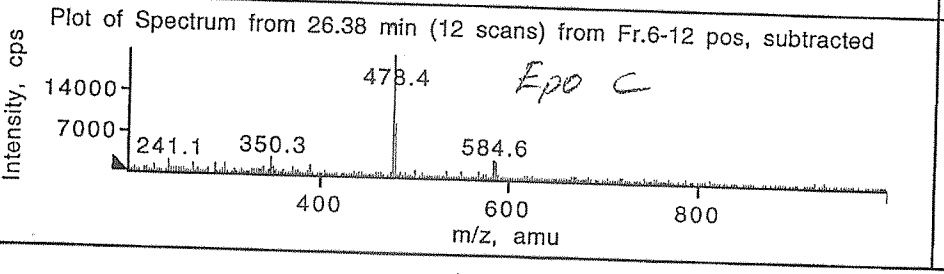

Plot of TIC from Fr.6-12 pos
Intensity, cps
6e6
3e6
3.5
5.5
12.2
15.7
19.6
23.5
26.3
32.6
35.0
38.4
49.9
6
12
18
24
30
36
42
48
Time, min

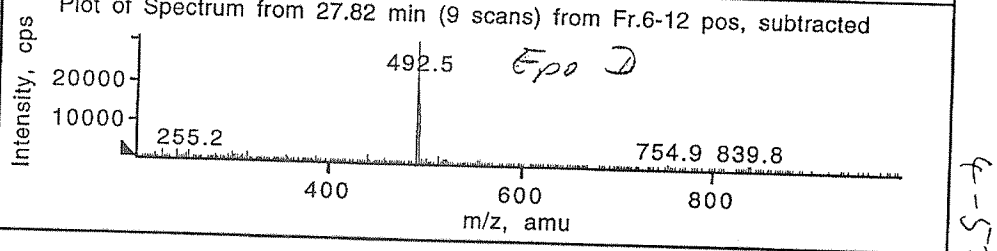

Plot of "Device A" from Fr.6-12 pos
40
20
1.6
6.7
9.6
12.1
19.5
20.4
23.2
26.3
32.5
38.5
50.8
6
12
18
24
30
36
42
48
Time, min


Plot of Spectrum from 19.55 min (7 scans) from Fr.6-12 pos, subtracted
Intensity, cps
4e5
2e5
494.4
Epo A
258.2
306.2
388.4
640.4
734.5
877.3
270
360
450
540
630
720
810
900
m/z, amu


Plot of Spectrum from 20.48 min (9 scans) from Fr.6-12 pos, subtracted
Intensity, cps
1.8e5
9.0e4
508.5
Epo B
316.2
806.9
400
600
800
m/z, amu


Plot of Spectrum from 23.31 min (10 scans) from Fr.6-12 pos, subtracted
Intensity, cps
2000
1000
256.0
341.3
464.3
522.5
635.4
877.4
400
600
800
m/z, amu


Plot of Spectrum from 26.38 min (12 scans) from Fr.6-12 pos, subtracted
Intensity, cps
14000
7000
478.4
Epo C
241.1
350.3
584.6
400
600
800
m/z, amu


Plot of Spectrum from 27.82 min (9 scans) from Fr.6-12 pos, subtracted
Intensity, cps
20000
10000
492.5
Epo D
255.2
754.9
839.8
400
600
800
m/z, amu

[redacted] Prep. RP-18 Chromatographie (Merck Prep-bar)

Soce 90 411 ca 30 g Probe wurde nicht getrocknet, da sie sich in MeOH schlecht löst.

LM M/W 6:4

Fraktion: 1-30 mit Gradientensprung auf M/W = 67/33

1	verworfen	
2	150 mg	414.
3	verworfen	
4		
5		
6	0,521 g	415.
7	1,892 g	~~416.~~
8	1,850 g	417.
9	1,847 g	418.
10		
11	1,486 g	419.
12	ca. 9,940 g	420.
13		
14	98 mg	421.
15	verworfen	
16		
17		
18	334 mg	422.
19	Gewicht = 232 mg, Eppe	423.
20		
21	500 mg	424.
22		

(250 mg Bristol, 200 mg Boehringer, 1,3 g Silica Fraktion verworfen)

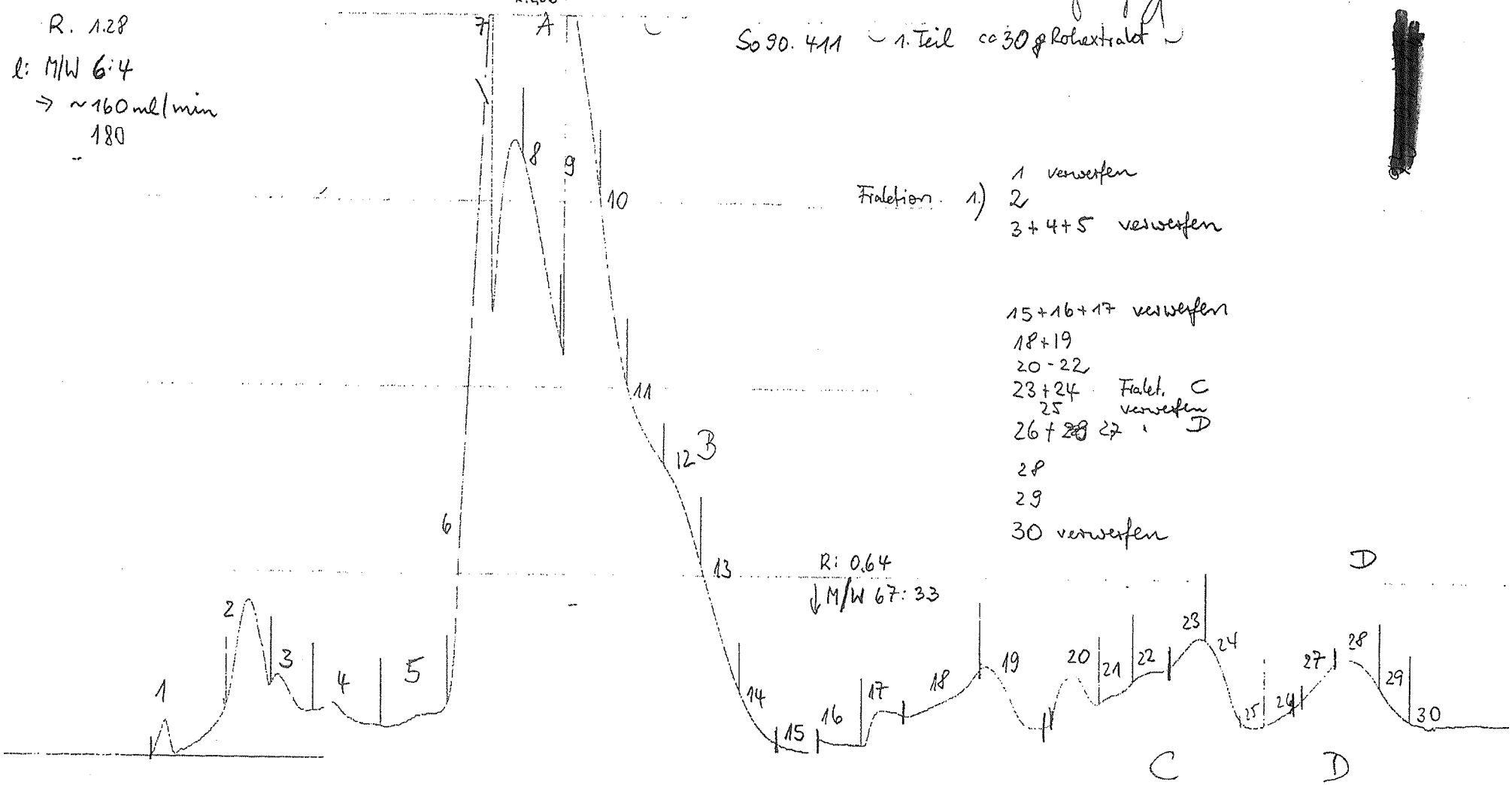
Fraktion		
~~22~~		
23	172 mg ~~5 [illegible] mg~~	425.
24	Gewicht = 153 mg	Epo C 426.
25	verworfen	
26 }	257 mg	427.
27 }		
28	Gewicht = 159 mg	Epo D 428.
29	Gewicht = 103 mg	429.
30	verworfen	

RP18 Chromatography

So 90.411 ~ 1. Teil ca 30 g Rohextrakt ~

R. 1.28
l: M/W 6:4
⇒ ~160 ml/min
180

R.256.



Fraktion: 1) 1 verwerfen
2
3+4+5 verwerfen

15+16+17 verwerfen
18+19
20-22
23+24 Fakt. C
25 verwerfen
26+~~28~~ 27 : D
28
29
30 verwerfen

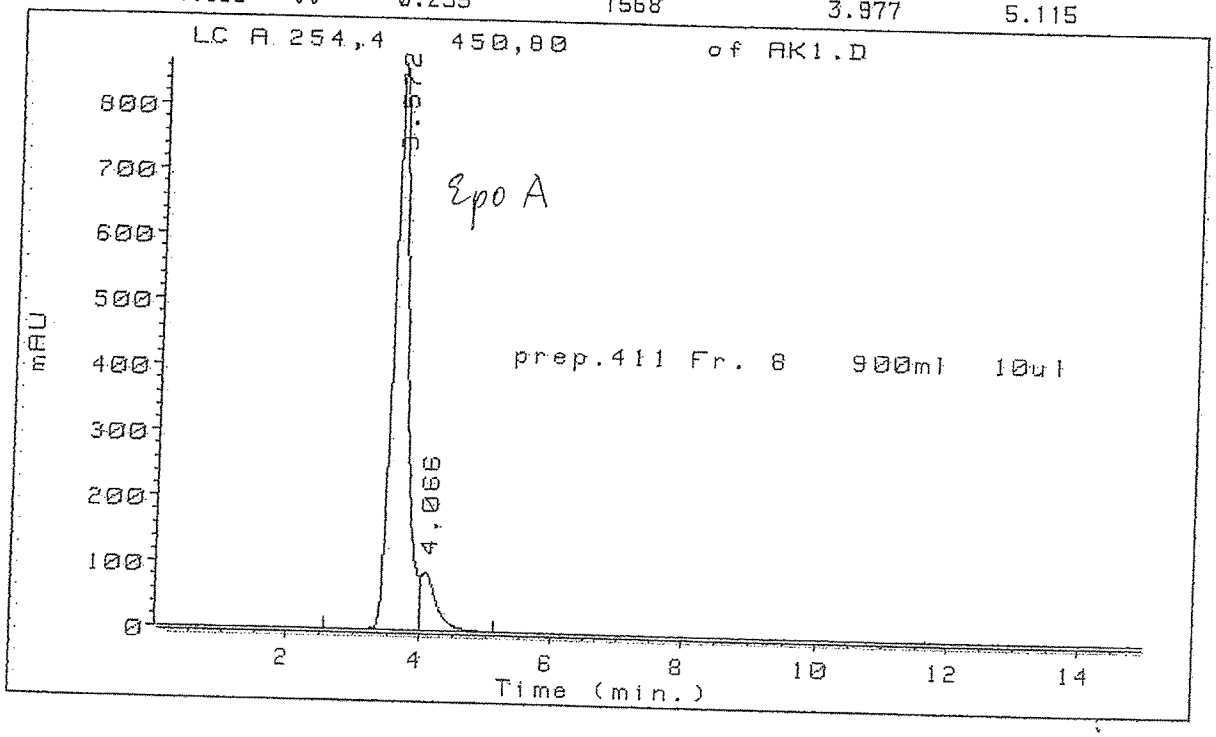
4-60

[redacted]

4-61

LC A 254,4 450,80 of AK1.D
DATA:AK1.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	3.572	BV	0.234	13828	2.544	3.977
2	4.066	VV	0.255	1568	3.977	5.115



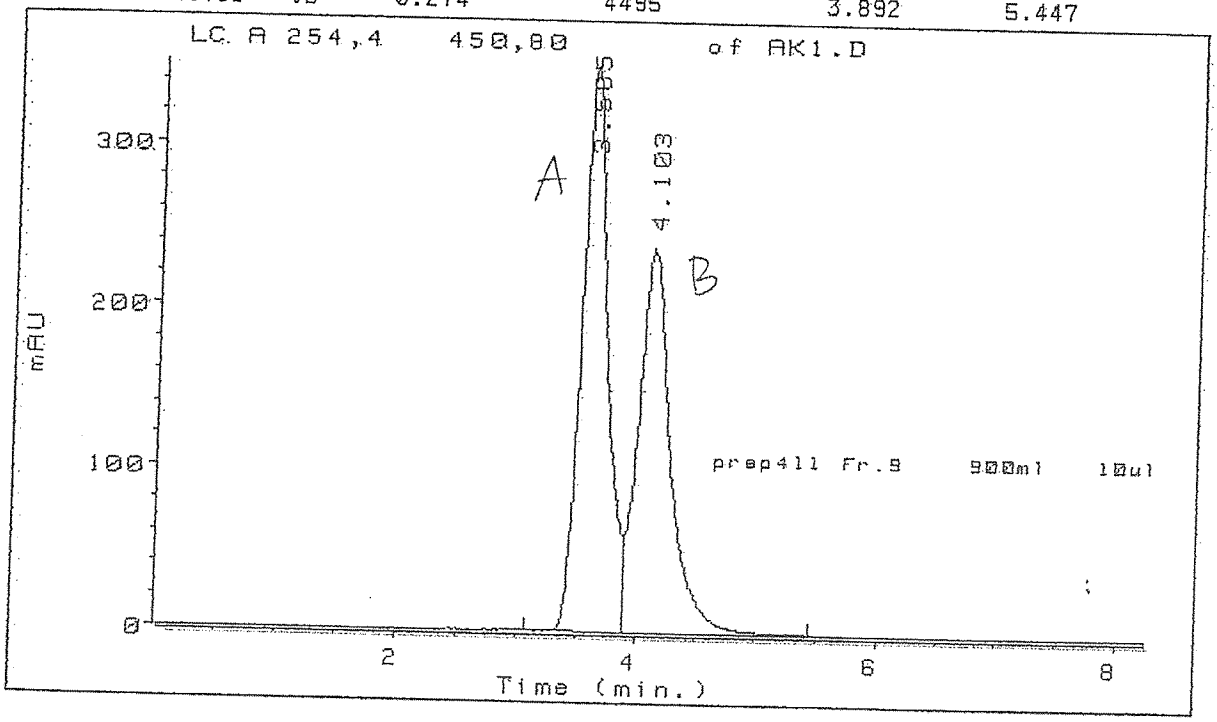
1,454 g A

0,164 g B

4-62

LC A 254,4 450,80 of AK1.D
DATA:AK1.D

Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	3.585	BV	0.227	5375	3.079	3.892
2	4.103	VB	0.274	4495	3.892	5.447

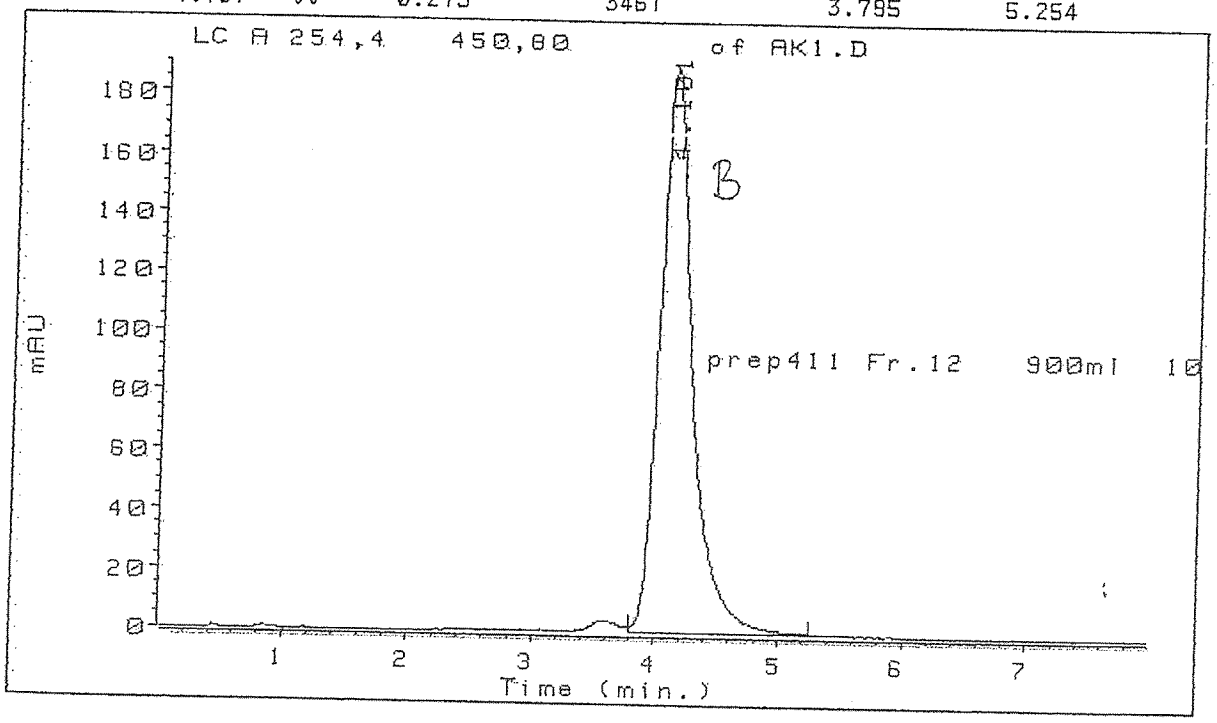


0,566g A

0,474g B

LC A 254,4 450,80 of AK1.D
DATA:AK1.D

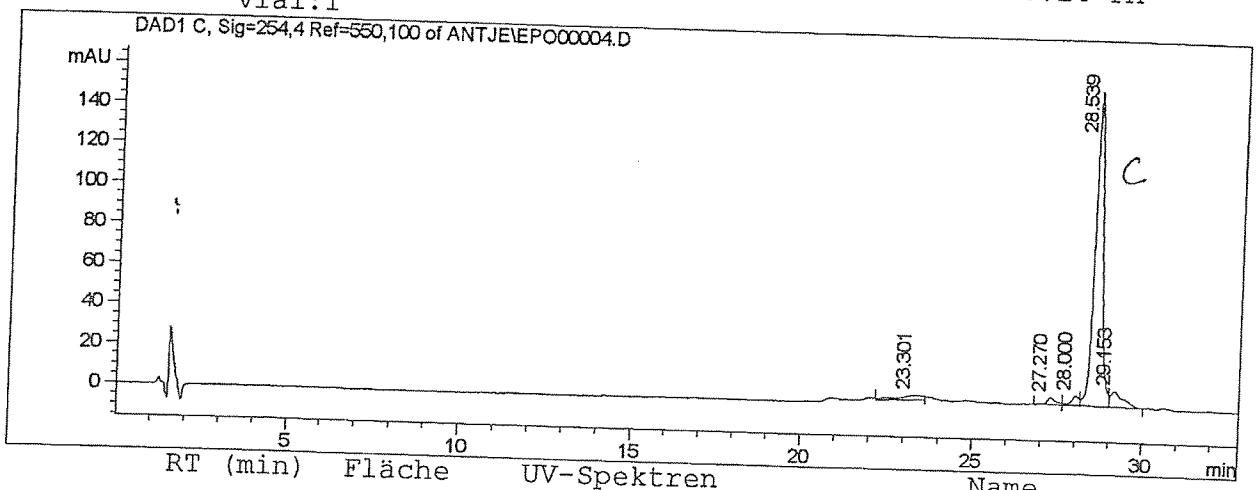
Peak#	Ret Time	Type	Width	Area	Start Time	End Time
1	4.101	VV	0.275	3461	3.795	5.254

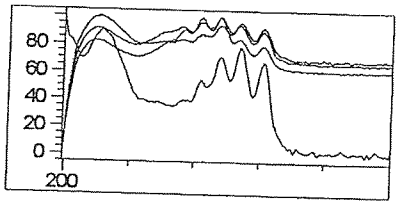
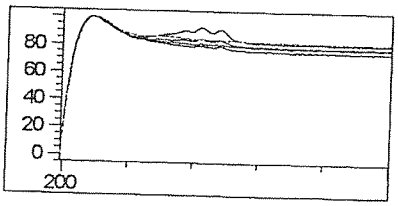
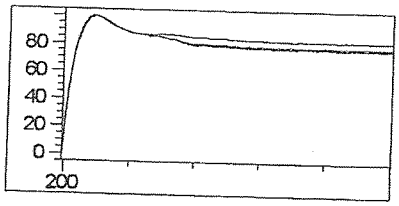
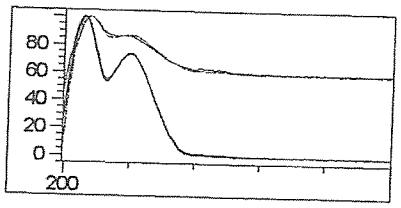


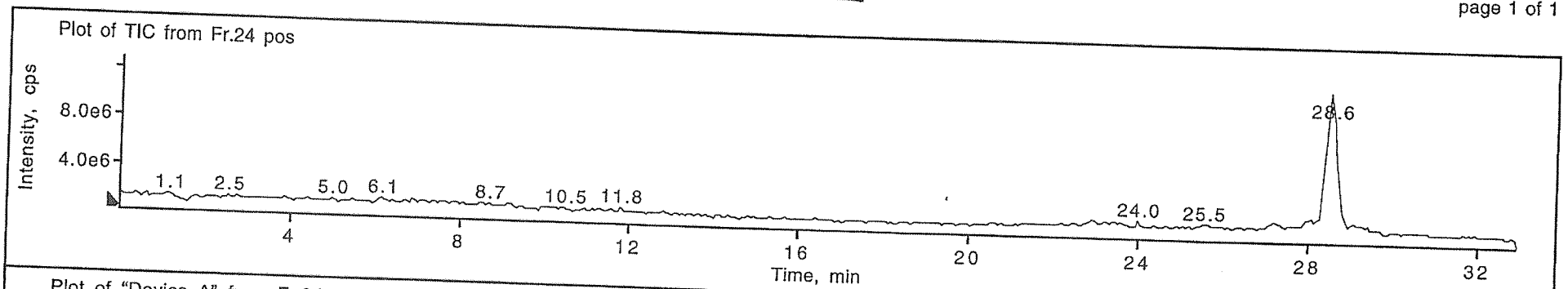
364mg B

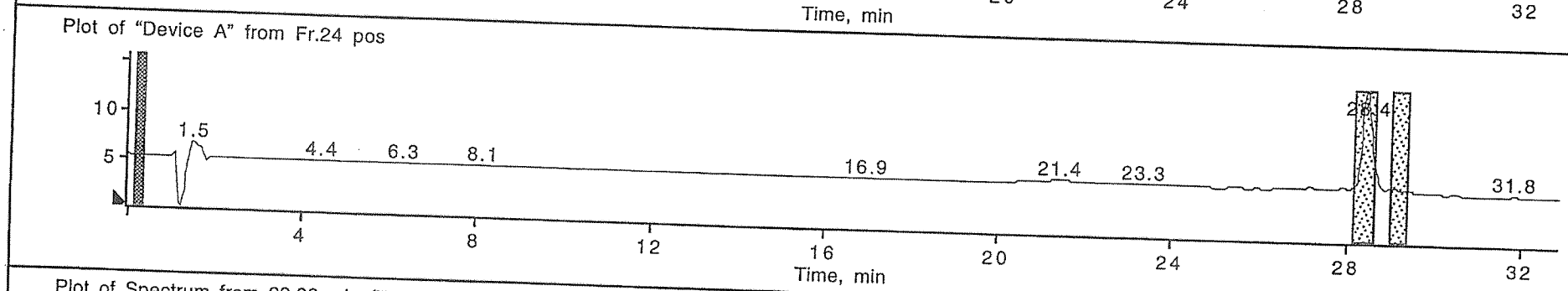
Report Layout (Original-File: Spectra.FRP)
Data File name: C:\HPCHEM\1\DATA\ANTJE\EPO00004.D
Method name: C:\HPCHEM\1\METHODS\SCREEN1.M
Sample Name: Fr.24
Injection Time: 2:40:26 PM
Sequence Name:
Report Style: screen1
data acquired by:Antje
vial:1

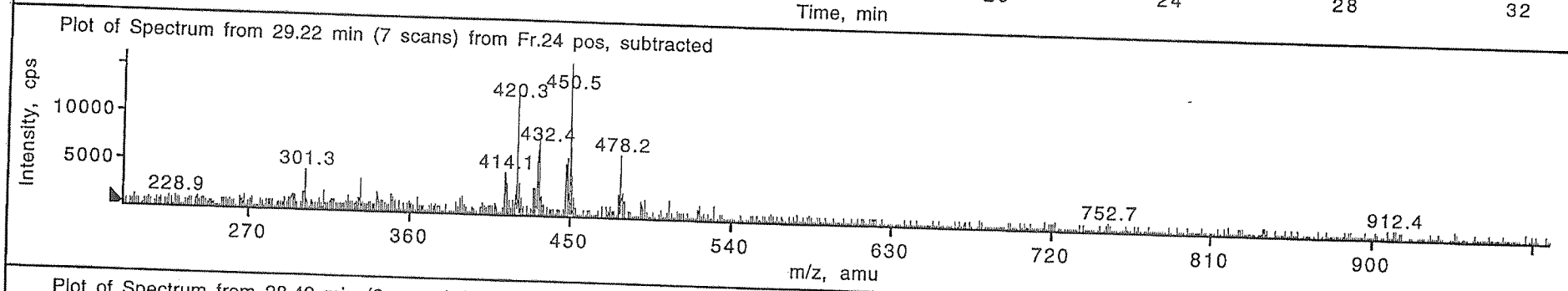
4-64
20µL
Sample Info: HPLC_MS_ ->
Screeninggradient
S090.426
on [redacted] 2:40:26 PM

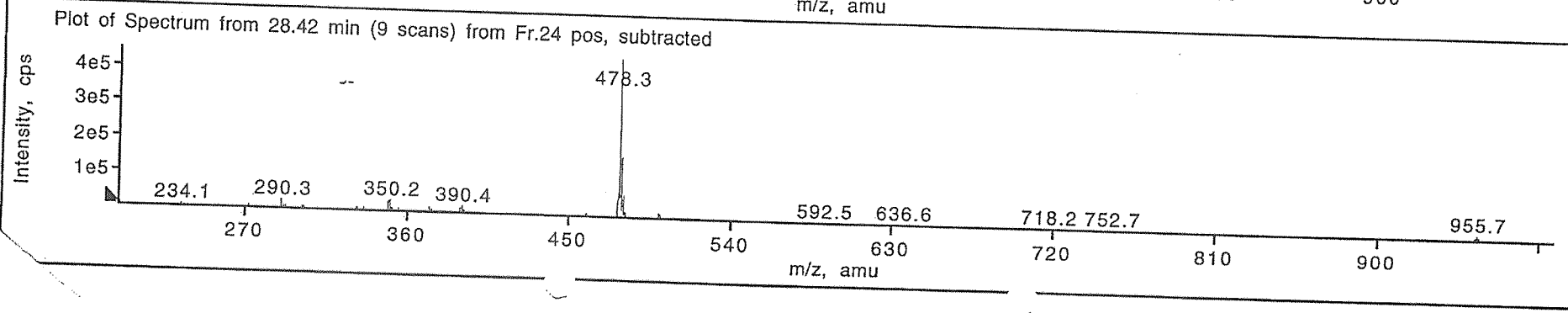


RT (min)	Fläche mAU*sec	UV-Spektren	Name
23.30	132.3		
27.27	66.4		
28.00	83.1		
28.54	2561.2		C


Plot of TIC from Fr.24 pos
Intensity, cps
8.0e6
4.0e6
1.1
2.5
5.0
6.1
8.7
10.5
11.8
24.0
25.5
28.6
4
8
12
16
20
24
28
32
Time, min


Plot of "Device A" from Fr.24 pos
10
5
1.5
4.4
6.3
8.1
16.9
21.4
23.3
28.4
31.8
4
8
12
16
20
24
28
32
Time, min


Plot of Spectrum from 29.22 min (7 scans) from Fr.24 pos, subtracted
Intensity, cps
10000
5000
228.9
301.3
414.1
420.3
432.4
450.5
478.2
752.7
912.4
270
360
450
540
630
720
810
900
m/z, amu


Plot of Spectrum from 28.42 min (9 scans) from Fr.24 pos, subtracted
Intensity, cps
4e5
3e5
2e5
1e5
234.1
290.3
350.2
390.4
478.3
592.5
636.6
718.2
752.7
955.7
270
360
450
540
630
720
810
900
m/z, amu

59-F

Report Layout (Original-File: Spectra.RPT)

Data File name: C:\HPCHEM\1\DATA\ANTJE\EPO00005.D

Method name: C:\HPCHEM\1\METHODS\SCREEN1.M

Sample Name: Fr.28

Sample Info: HPLC_MS_ ->

Injection Time: 3:27:54 PM

Sequence Name:

Report Style: screen1

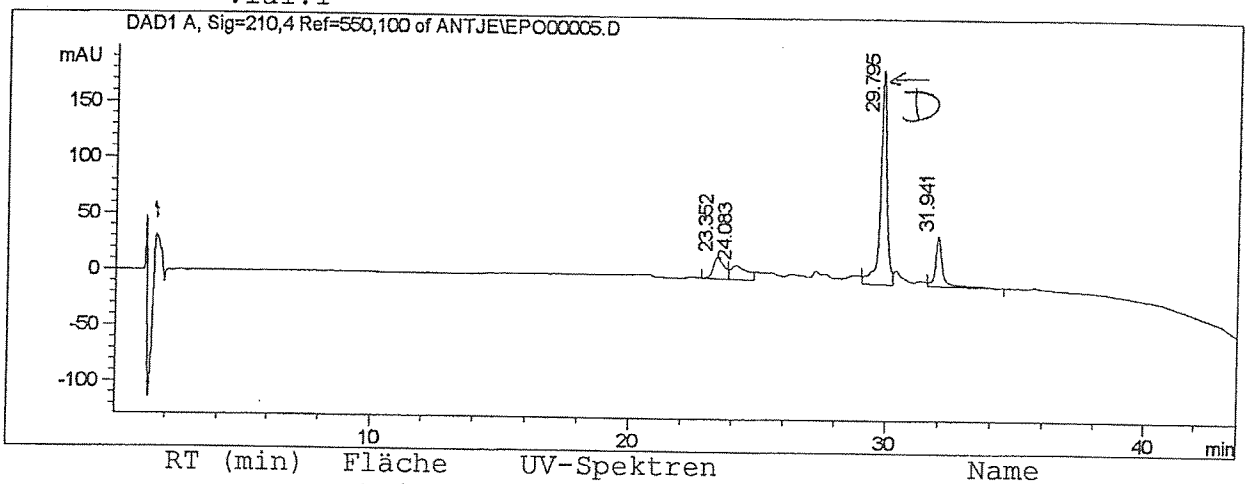
data acquired by:Antje

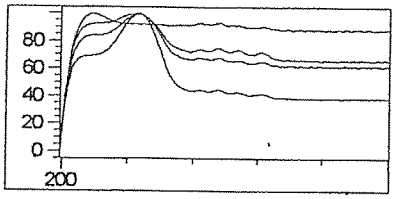
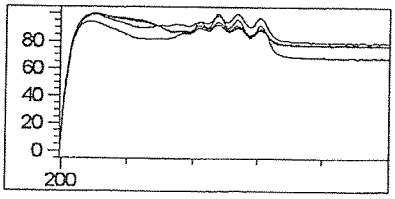
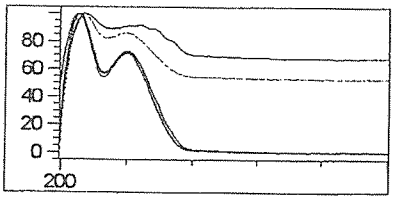
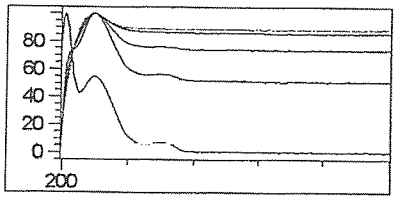
vial:1

on: [redacted] 3:27:54 PM

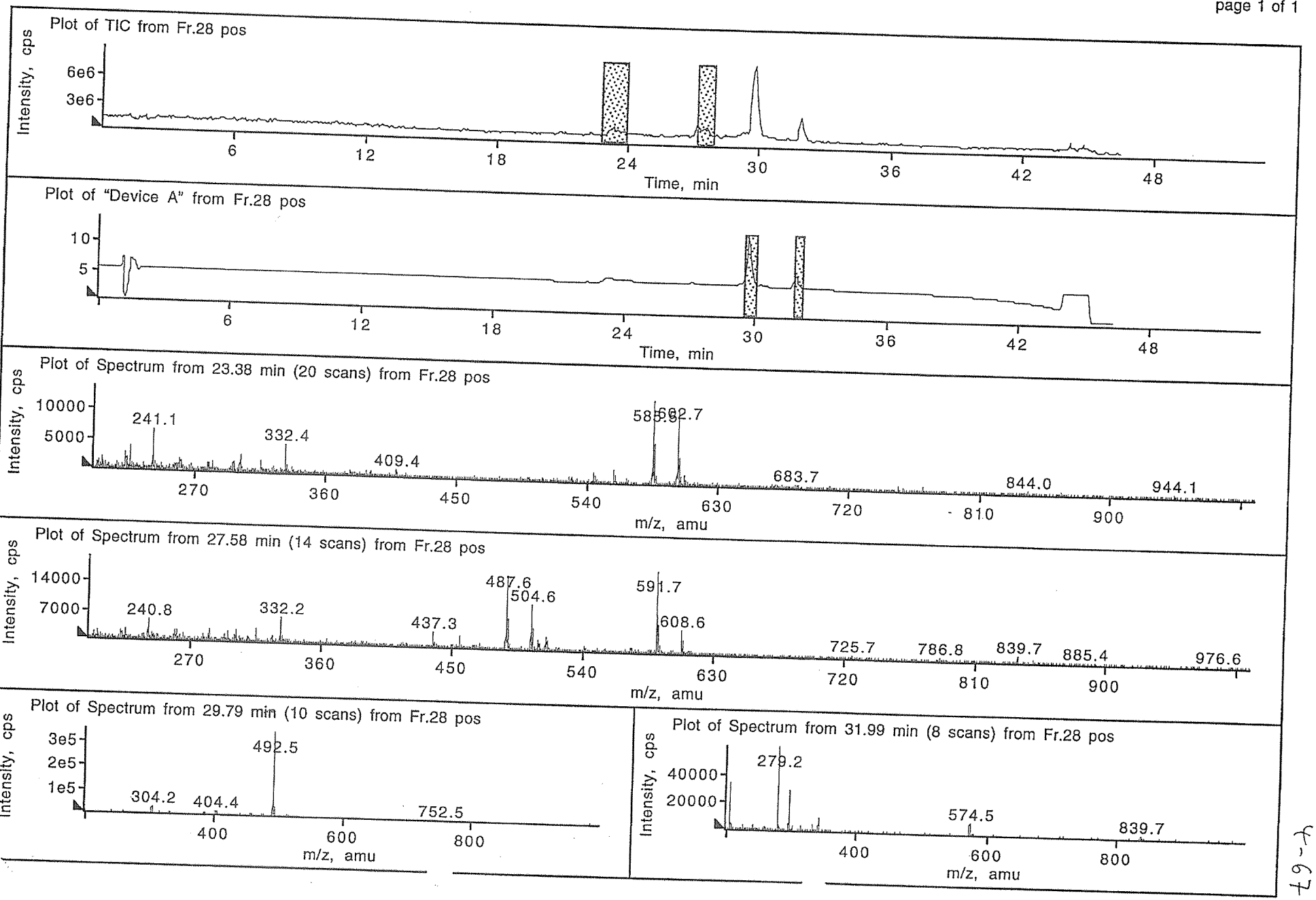
4-66

So 90.428

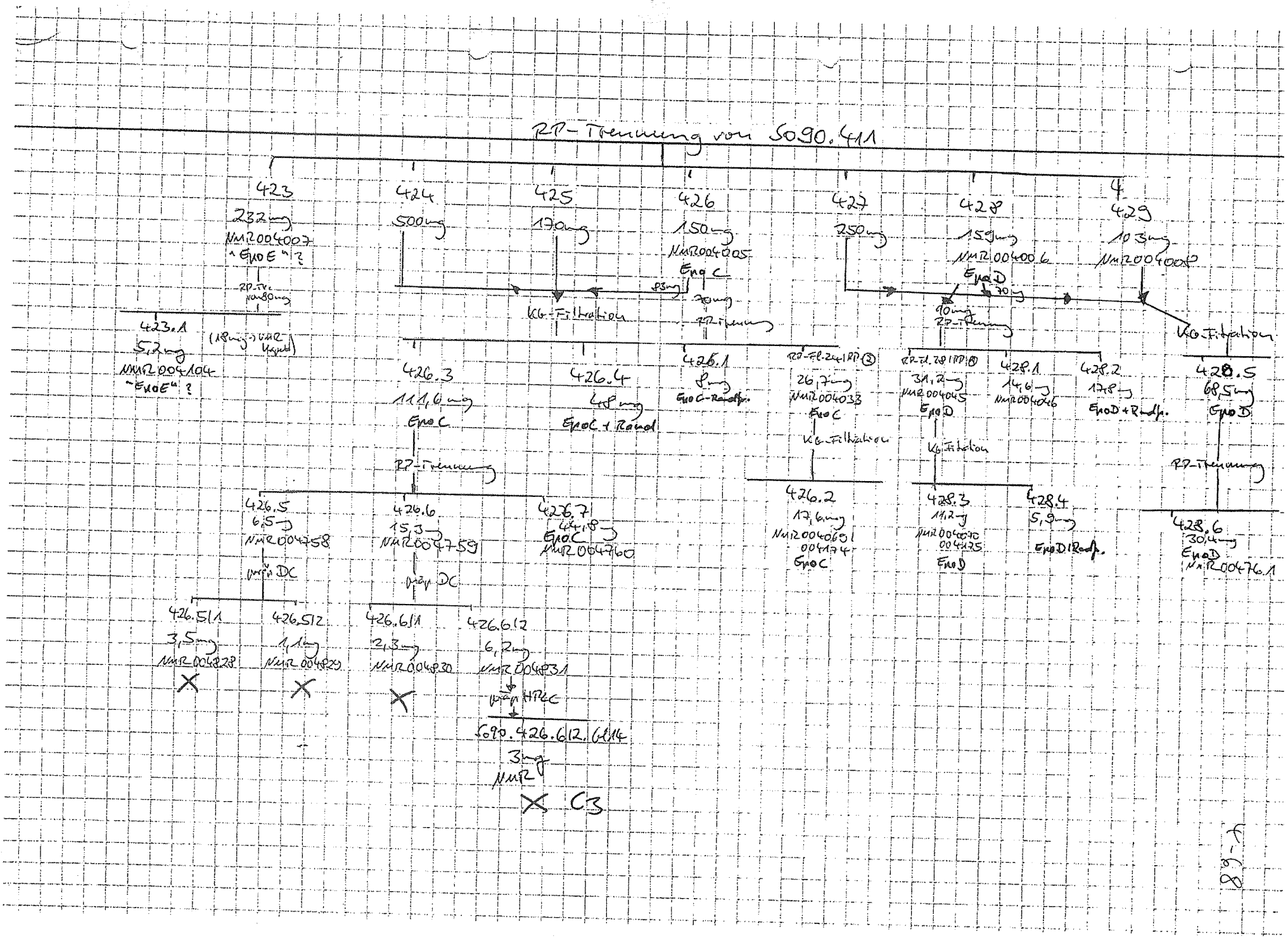


RT (min)	Fläche mAU*sec	UV-Spektren	Name
23.35	607.2		
24.08	573.3		
29.80	3346.7		D
31.94	906.1		E2

Created on: [redacted] 4:12:24 PM


Plot of TIC from Fr.28 pos
Intensity, cps
6e6
3e6
6
12
18
24
30
36
42
48
Time, min
Plot of "Device A" from Fr.28 pos
10
5
6
12
18
24
30
36
42
48
Time, min
Plot of Spectrum from 23.38 min (20 scans) from Fr.28 pos
Intensity, cps
10000
5000
241.1
332.4
409.4
583.6
662.7
683.7
844.0
944.1
270
360
450
540
630
720
810
900
m/z, amu
Plot of Spectrum from 27.58 min (14 scans) from Fr.28 pos
Intensity, cps
14000
7000
240.8
332.2
437.3
487.6
504.6
591.7
608.6
725.7
786.8
839.7
885.4
976.6
270
360
450
540
630
720
810
900
m/z, amu
Plot of Spectrum from 29.79 min (10 scans) from Fr.28 pos
Intensity, cps
3e5
2e5
1e5
304.2
404.4
492.5
752.5
400
600
800
m/z, amu
Plot of Spectrum from 31.99 min (8 scans) from Fr.28 pos
Intensity, cps
40000
20000
279.2
574.5
839.7
400
600
800
m/z, amu

4-67


RP-Trennung von 5090.411
423
232mg
NMR004007
"EtoE"?
RP-Tr.
423.1
5,2mg
NMR004104
"EtoE"?
424
500mg
425
170mg
426
150mg
NMR004005
EtoC
427
250mg
428
159mg
NMR004006
EtoD
429
103mg
NMR004007
VK-Filtration
426.1
8mg
EtoC-Reinfr.
426.3
111,6mg
EtoC
RP-Trennung
426.4
48mg
EtoC + Reinol
426.5
6,5mg
NMR004758
426.6
15,3mg
NMR004759
426.7
44,8mg
EtoC
NMR004760
426.511
3,5mg
NMR004828
426.512
1,1mg
NMR004829
426.611
2,3mg
NMR004830
426.612
6,2mg
NMR004831
HPLC
5090.426.612.6114
3mg
NMR
C3
RP-Fl.24 RP③
26,7mg
NMR004033
EtoC
426.2
17,6mg
NMR004060
004174
EtoC
RP-Fl.28 RP①
31,2mg
NMR004045
EtoD
428.1
14,6mg
NMR004046
428.2
17,8mg
EtoD + Reinfr.
428.3
11,2mg
NMR004030
004125
EtoD
428.4
5,9mg
EtoD Reinfr.
428.5
68,5mg
EtoD
428.6
30,4mg
EtoD
NMR004761

[redacted]

RP-Trennung von RP-Fl. 24 (70 mg) (von 153 mg)
in 2 Läufen 4-69

Säule = Nucleosil C18, 7 µm , 20 × 250 mm

LM = 70 MeOH, 30 Na_2HPO_4, 0,01 M, pH 7,0 → umgestellt auf 73 MeOH, 27 Na_2HPO_4, 0,01 M, pH 7,0

Pumpe: 200 , Papier: 5 mm/min

λ = 254 nm , Range: 0,64 - ∞

Fraktionen bis zur H_2O-Phase eingeengt, 2× mit EE extrahiert, EE-Phase mit H_2O gewaschen und mit $MgSO_4$ getrocknet.

Fraktionierung:

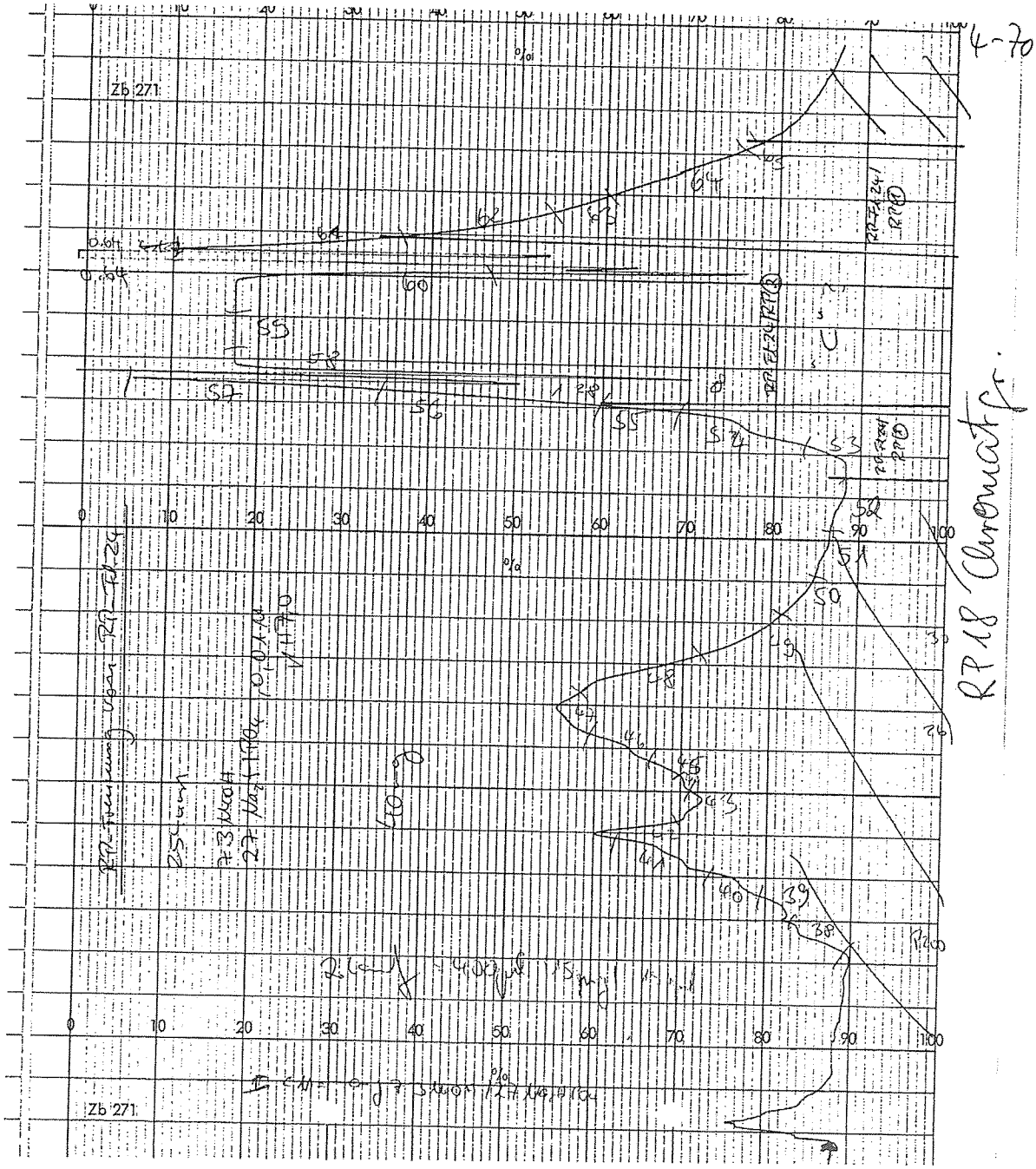
~~RP-Fl. 24/RP-①~~ = Randfr.: Gewicht: 8 mg → Ansatz Epo (117)
426.1

~~RP-Fl. 24/RP-② = Peak: C~~ " = 26,7 mg, NMR 004033, ¹³C, Nr. 132 gelb
→ VG-Filtration (5.11.96)

→ ~~2/9~~ Ep (84)

DC: 95 CH_2Cl_2 / 5 MeOH

Leergewicht: RP-① = 19,3964 g
RP-② = 27,7225 g


4-70
RP18 Chromatogr.
Zb 271
%
0
10
20
30
40
50
60
70
80
90
100

silica gel

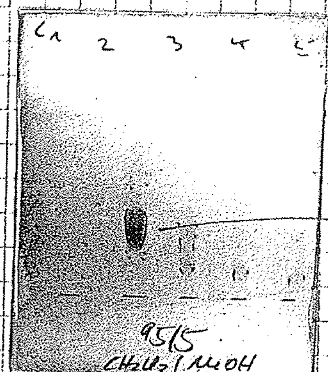
KG-Filtration von So 90.411/RP-Fl.24/RP-② ≙ "C" [redacted]

So 90.411/RP-Fl.24/RP-② = 26,7mg in CH_2Cl_2 aufgetragen

offene Säule = Material = KG60 , 0,063mm - 0,200mm

I 8cm Höhe , Ø 0,9cm

→ naß gepackt in 97 CH_2Cl_2 / 3 MeOH , eluiert mit 97 CH_2Cl_2 / 3 MeOH



Epo C

angesprüht mit Vanillin / H_2SO_4

Fraktionierung:

So 90.411/RP-Fl.24/RP-②/KG = Gewicht: 17,6mg , ≙ A 5mg
426.2
C

DMSO-d6 ?
NMR 004174 ,pl. 273 gelb
NMR 004069
Ue. 167 gelb

0,2mg Testlösung

<1,2mg Epo 84 ; 10mg Epo 85 ; 2mg Epo 86 ; 2mg Aus 88

Epo C MTC = 50ug/ml

Leergewicht: 19,4406g

RP-Trennung von RP-Fl. 28 (90mg von 159mg)

So 90.411 RP-Fl. 28 = 90mg von 159mg in 3 Läufen getrennt

Säule = Merckoil C18, 9µm, 20 × 250mm

Lm = 75 MeOH / 25 Na_2HPO_4 / 0,01 M, pH 7,0

Pumpe = 200, Papier = Sum/Min

λ = 254nm, Range = 0.64 - ∞

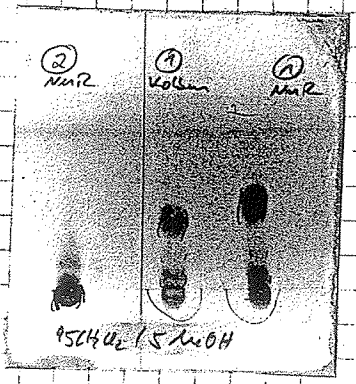
Fraktionen mit zur H_2O-Phase eingengt, 3× mit EE extrahiert, EE-Phase mit H_2O gewaschen und mit $MgSO_4$ getrocknet.

Fraktionierung:

So 90.411 RP ~~Fl.28~~ ① / RP-① = 31,2mg, NMR 004045, Nr. 145 gIG
13C UG = Filtration (S. U. 96)

So 90.411 RP-Fl.28 / RP-② = 14,6mg, NMR 004046, Nr. 146 gIG
? 428.1

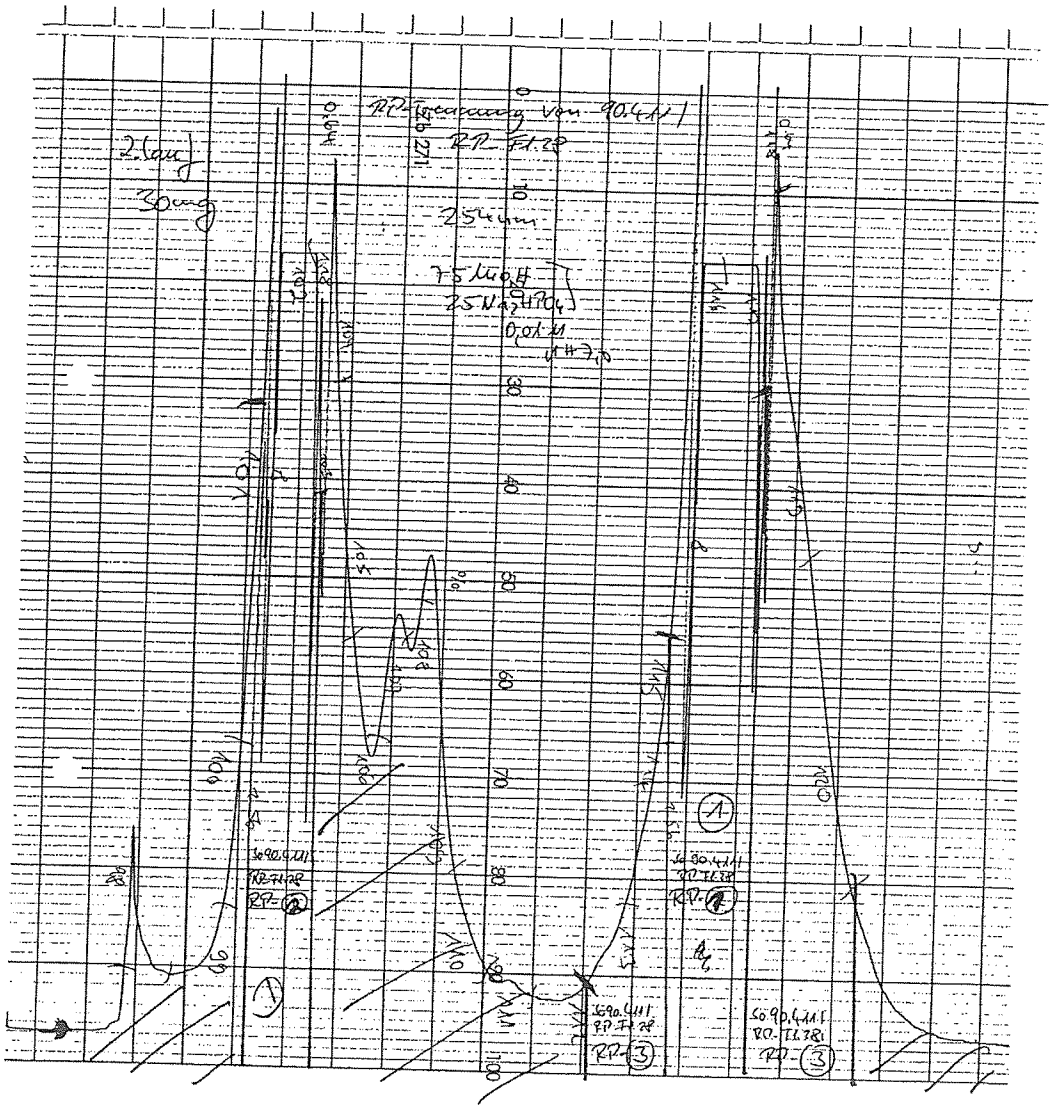
So 90.411 RP-Fl.28 / RP-③ = 17,8mg
428.2 Rechromatographie



Vergleichbar: RP-① = 22,1899g

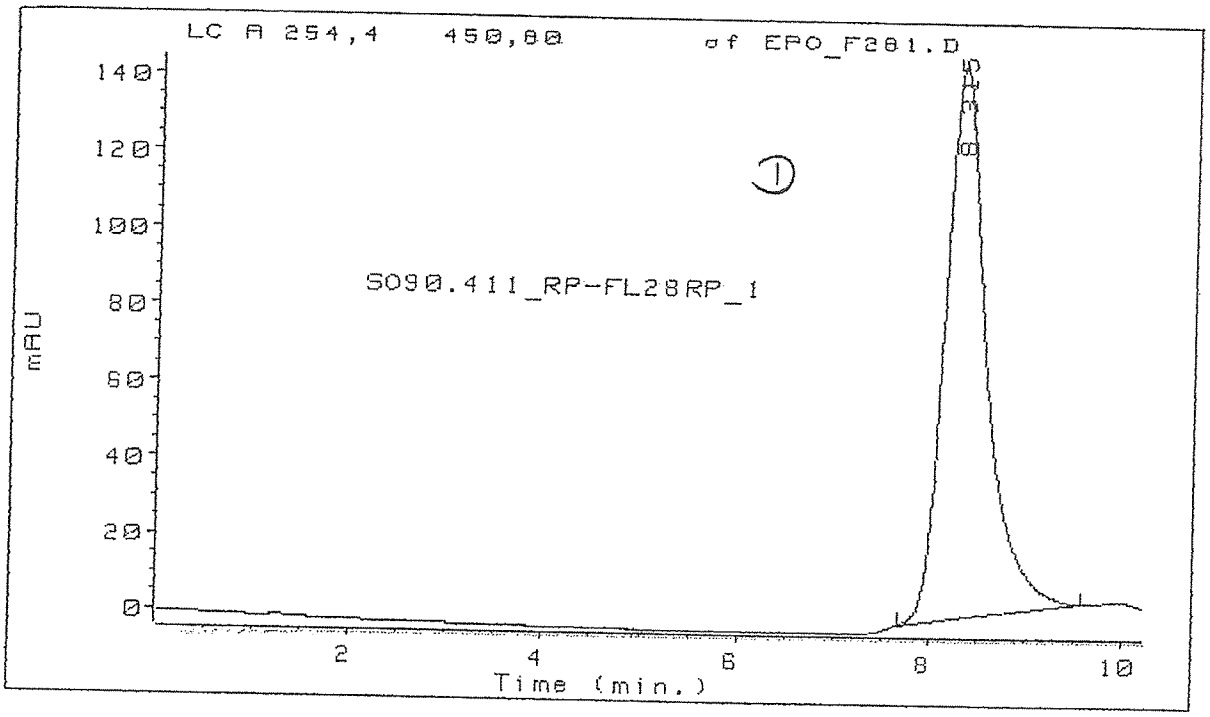
RP-② = 17,9169g

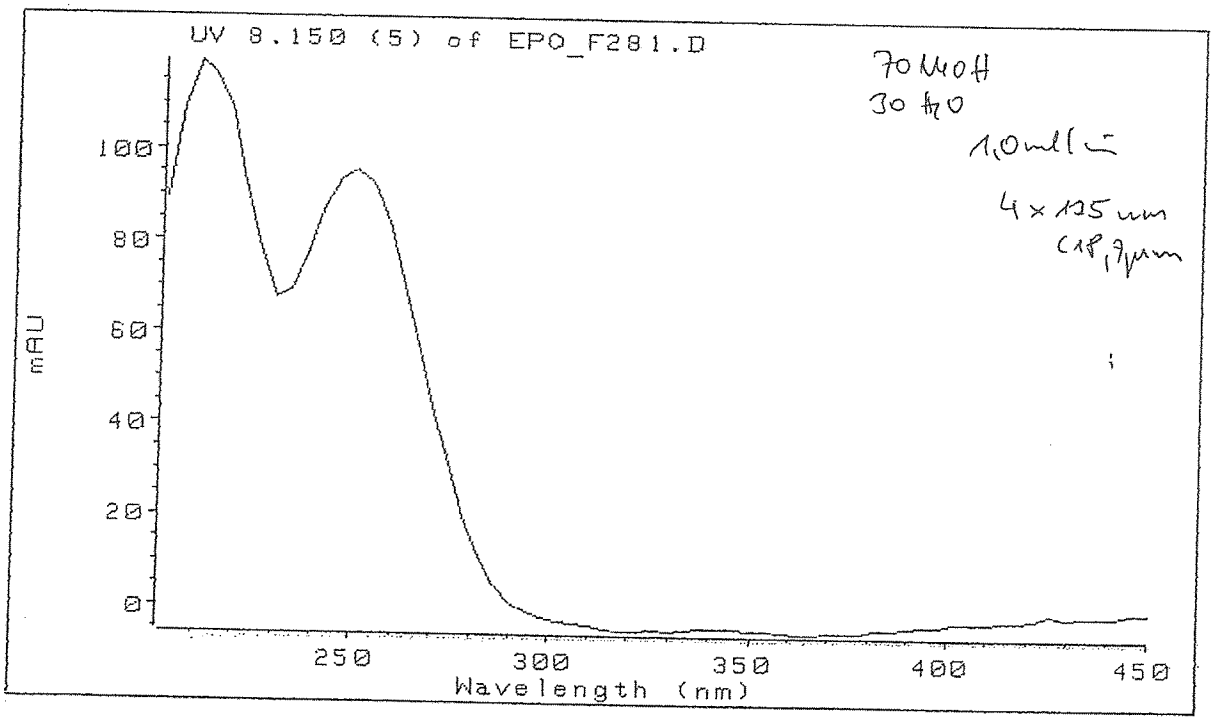
RP-③ = 16,9307g



D

RP18 chromatogr.





70 MeOH
30 H2O
1,0 ml/min
4 x 125 mm
C18, 5 µm

Silica gel

KG-Filtration von So 90.411 /RP-Fl.28 /RP-① = "D" [redacted]

4-75

So 90.411 /RP-Fl.28/RP-① = 31,2 g in CH_2Cl_2 aufgetragen

offene Säule = Material = KG 60, 0,063 mm - 0,2 mm

18 cm Höhe, Ø 0,9 cm

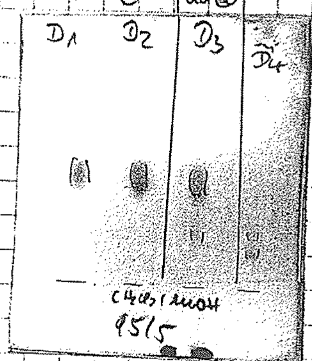
-> naß gepackt in 97 CH_2Cl_2 / 3 MeOH, eluiert mit

97 / 3 MeOH

428.3 428.4

KG-① KG-②

Epo D



angefärbt -> Vanillin/H_2SO_4

Fraktionierung =

So 90.411/RP-Fl.28/RP-①/KG-① = (1.1-2) = Gewicht = ~~11,2~~ 13 mg -> dwe d6; NMR 004175; 274 g/l
428.3 D

0,1 g Tartsäure ... ; NMR 004070; ... 168 g/l

2 mg Epo/22.5 g ... Am 87 ... Am 85

~~So 90.411/RP-Fl.28/RP-①/KG-② = 61.3 = Gewicht = 5,9 mg -> verworfen~~
~~428.4 D Anreicherung~~

EpoD = MIC = 15 ug/ml

Leergewicht = 17,749 g KG-①

4,784 g KG-②

Einlieferungsdatum: [redacted]

Spektren-Nr.: 004033

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

132.

4 - 78

Substanz-Bez.: 590.411 IRP-F124 IRP-(2) ^{13}C

Summenformel:

Substanzhersteller: Polan

Abteilung: MC (1.1.7) Tel.: 343

Kernart (^{1}H, ^{13}C, ^{31}P, andere?)

Substanz-Menge: 20 mg, Molmasse:

geeignetes Lösungsmittel: CD_3OD weitere Messung nach Zugabe von

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒
im Tiefkühlfach ☐
im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen δ = 0 und 9

Gewünscht: nur Spektrum ☐
plus Integral ☒
Interpretation ☐

Zahl der Akkumulationen (falls > 104):

Art des Experiments

^{1}H Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en):

^{13}C ^{1}H-Entkopplung:

Breitband ☒ selektiv ☐

DEPT ☒ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

Linienausdruck ☐

^{1}H

δ = 8.9 bis −0.1 (0.15 ppm/cm) ☒

11.9 bis −0.1 (0.2 ppm/cm) ☐

Drehungen:

10 Hz/cm ☐ von δ = bis

^{13}C normal (δ = 220 bis 0) ☒

anderes Format:

Sonderwünsche: COSY ☐ ^{13}C — ^{1}H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☒ AM-300 ☐ ARX-400 ☐ DMX-600

gespeichert unter Nr. SI2[illegible]33/10, 12, 10

Bitte um Rücksprache ☐

Kommentar:

(Unterschrift)

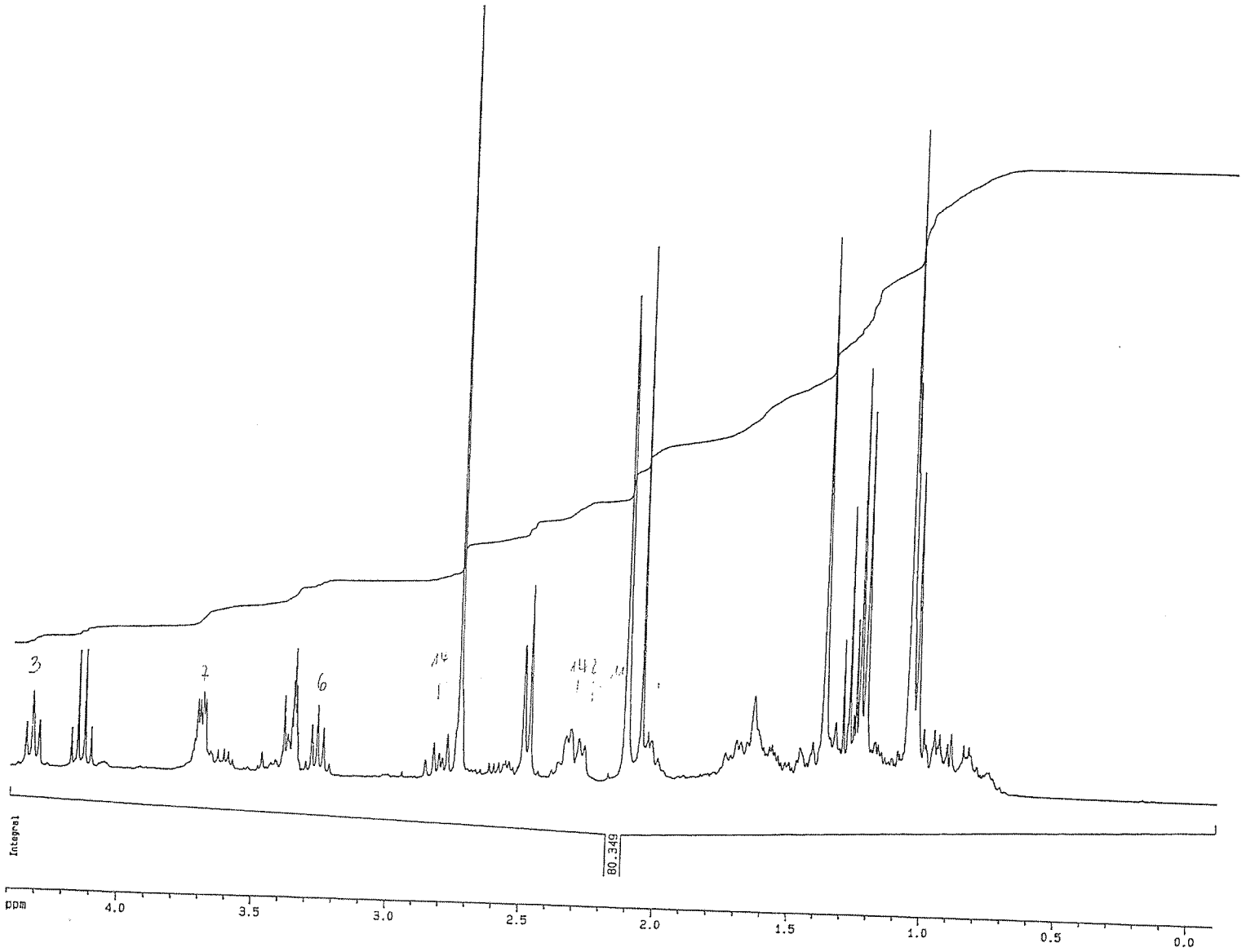
Epothilon

So 90.411 / RP-FL-24 / RP-②

426

20mg → 26,7mg

SIPZ4033 10 1 Pohlan



Current Data Parameters

NAME	SIPZ4033
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	13.33
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	88
DS	0
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	128
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	15.00 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-4.00 dB

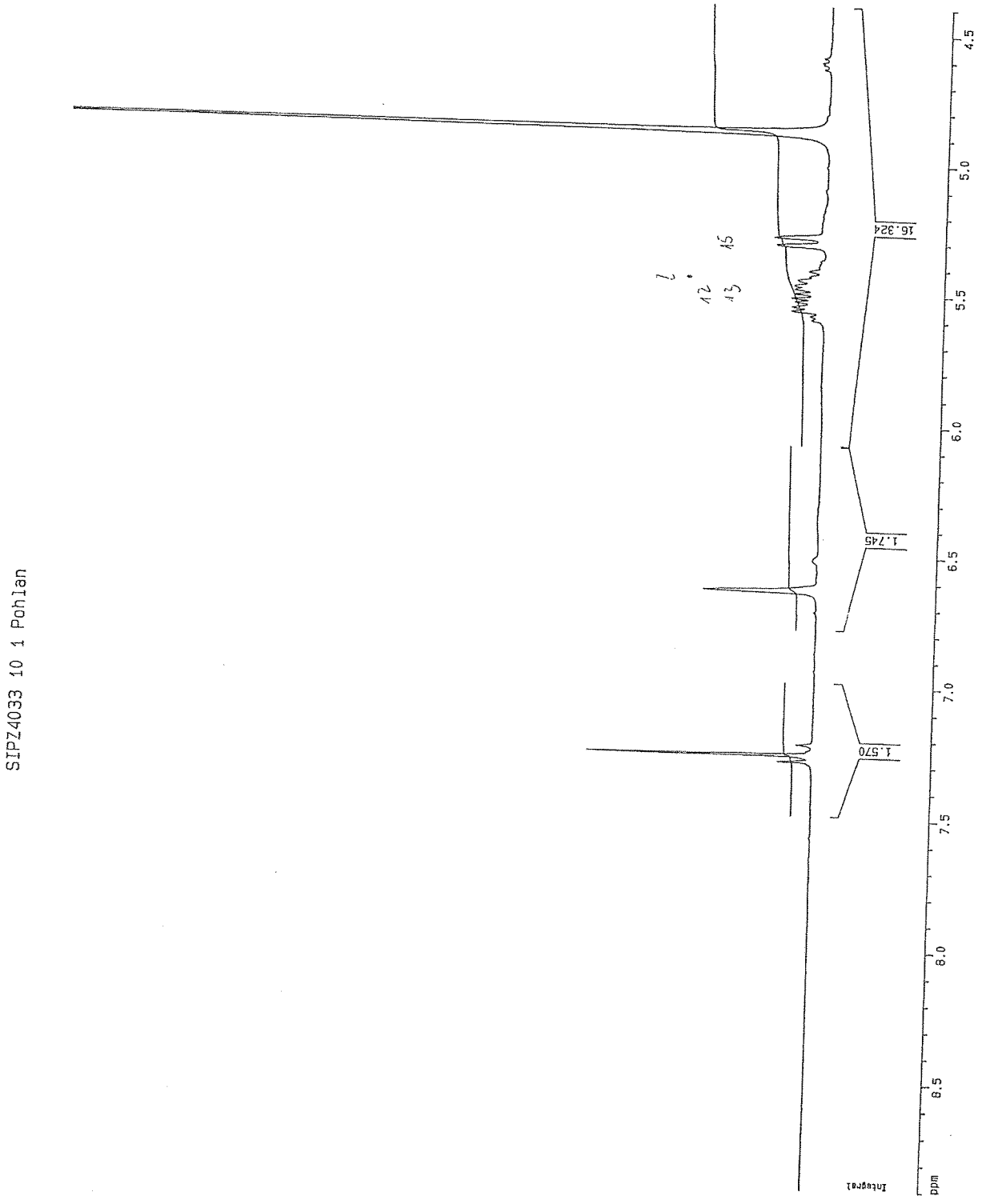
F2 - Processing parameters

SI	16384
SF	300.1299924 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm

47-29


SIPZ4033 10 1 Pohlan
Integral
1.570
1.745
16.324
ppm
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5

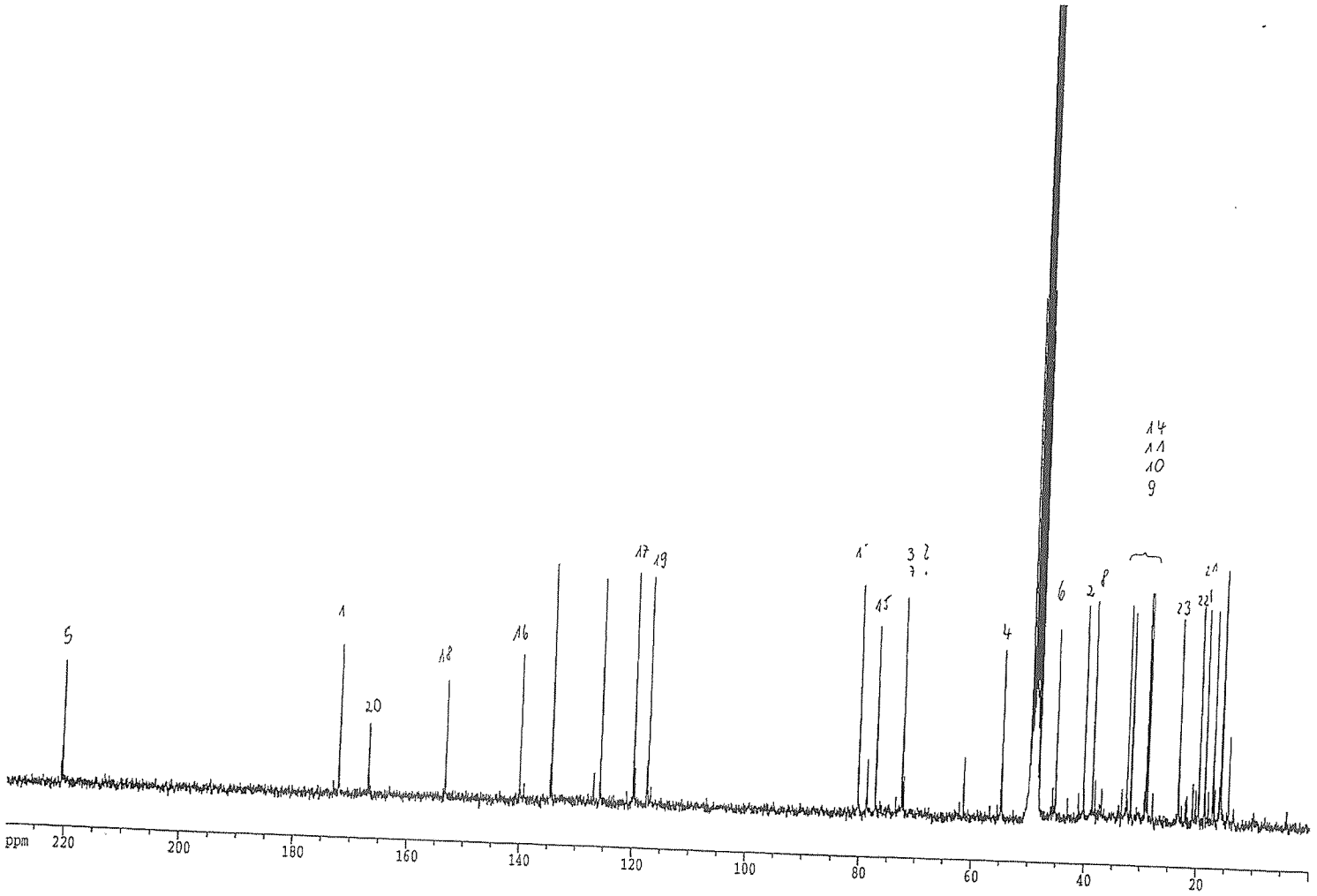
Epothilon

So 90.411 IRP - 71.24 IRP. (α)

1424

" C "

SIPZ4033 20 1 Pohlan



Current Data Parameters

NAME	SIPZ4033
EXPNO	20
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	18.21
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zgpg30
TD	32768
SOLVENT	MeOH
NS	8000
DS	2
SWH	23809.523 Hz
FIDRES	0.726609 Hz
AQ	0.6881780 sec
RG	2298.8
DW	21.000 usec
DE	5.50 usec
TE	300.0 K
D12	0.00002000 sec
PL13	25.00 dB
D1	1.00000000 sec
CPDPRG2	waltz16
PCPD2	97.00 usec
SFO2	300.1312005 MHz
NUC2	1H
PL2	-3.00 dB
PL12	12.20 dB
P1	11.00 usec
DE	5.50 usec
SFO1	75.4772501 MHz
NUC1	13C
PL1	-3.00 dB
D11	0.03000000 sec

F2 - Processing parameters

SI	32768
SF	75.4676419 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	30.00 cm
F1P	230.000 ppm
F1	17357.56 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	7.66667 ppm/cm
HZCM	578.58527 Hz/cm

4-84

/u/data/chk/nmr/SIPZ4033/20/pdata/1/screen Tue [redacted] 59:03 [redacted]

DU=u, USER=chk, NAME=SIPZ4033, EXPNO=20, PROCNO=1
F1=230.000ppm, F2=0.000ppm, MI=0.00cm, MAXI=10000.00cm, PC=1.400

4-82

#	ADDRESS	FREQUENCY [Hz]	[PPM]	INTENSITY
1	6711.5	16634.855	220.4237 C 5	2.95
2	11729.2	12988.931	172.1126 C 1	3.44
3	12268.5	12597.046	166.9198 - 20	1.71
4	13677.1	11573.543	153.3577 - 18	2.70
5	15059.4	10569.200	140.0494 - 16	3.46
6	15615.0	10165.478	134.6998	0.86
7	15640.6	10146.885	134.4534 -	5.61
8	16421.5	9579.455	126.9346	0.70
9	16539.5	9493.721	125.7986 -	5.35
10	17145.8	9053.167	119.9609 - 17	5.34
11	17174.2	9032.517	119.6873	0.84
12	17398.9	8869.258	117.5240	0.88
13	17411.1	8860.417	117.4068 - 19	5.22
14	21268.5	6057.571	80.2671 -	5.41
15	21435.0	5936.585	78.6640	1.22
16	21589.0	5824.736	77.1819 - 15 l	4.28
17	22074.7	5471.765	72.5048	5.04
18	22109.2	5446.690	72.1725	0.87
19	23214.8	4643.369	61.5279	1.35
20	23911.4	4137.193	54.8208 - 4	4.02
21	24427.1	3762.479	49.8555	11.90
22	24456.7	3741.021	49.5712	34.54
23	24486.1	3719.640	49.2879	70.13
24	24515.7	3698.156	49.0032	80.32
25	24545.1	3676.791	48.7201	69.03
26	24574.6	3655.367	48.4362	34.30
27	24604.0	3633.971	48.1527	12.04
28	24859.0	3448.694	45.6977	0.72
29	24909.2	3412.198	45.2141 - 6	4.38
30	25330.7	3105.970	41.1563	0.62
31	25423.8	3038.285	40.2594 - 2	4.95
32	25580.7	2924.324	38.7494 - 8	5.13
33	25632.7	2886.490	38.2481	0.92
34	25754.1	2798.305	37.0795	0.73
35	26126.9	2527.458	33.4906	0.74
36	26224.1	2456.771	32.5540	4.97
37	26298.1	2403.007	31.8416	4.80
38	26550.6	2219.588	29.4111	0.72
39	26578.0	2199.672	29.1472	5.24
40	26607.5	2178.218	28.8629	5.45
41	26699.7	2111.245	27.9755	0.68
42	27183.1	1760.011	23.3214 - 23	4.69
43	27335.5	1649.258	21.8538	0.63
44	27438.5	1574.374	20.8616	0.96
45	27497.0	1531.911	20.2989	0.76
46	27551.4	1492.387	19.7752 - 22	5.09
47	27665.8	1409.260	18.6737 - 21	4.97
48	27735.3	1358.757	18.0045	0.76
49	27819.7	1297.446	17.1921 - 24	4.98
50	27855.0	1271.734	16.8514	0.80
51	27928.3	1218.500	16.1460	0.88
52	27951.2	1201.869	15.9256	5.26
53	27965.0	1191.823	15.7925	5.81
54	28102.7	1091.776	14.4668	2.05

Einlieferungsdatum: [redacted]

Spektren-Nr.: ~~004069~~ 4-84

167.

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

Substanz-Bez.: Jo 90.4.11 RP_Fl. 24 / RP-2 / KG=C

Summenformel:

Substanzhersteller: Polan

Abteilung: MC (1.1.2) Tel.: 343

Kernart (¹H) ¹³C, ³¹P, andere?)

Substanz-Menge: 17,6 mg, Molmasse:

geeignetes Lösungsmittel: CD3OD weitere Messung nach Zugabe von

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒
im Tiefkühlfach ☐
im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen δ = 0 und 9

Gewünscht: nur Spektrum ☒
plus Integral ☒
Interpretation ☐

Zahl der Akkumulationen (falls > 104):

Art des Experiments

[¹H] Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en):

[¹³C] ¹H-Entkopplung:

Breitband ☐ selektiv ☐

DEPT ☐ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

[¹H]

δ = 8.9 bis −0.1 (0.15 ppm/cm) ☒

11.9 bis −0.1 (0.2 ppm/cm) ☐

Linienausdruck ☐

Drehungen:

10 Hz/cm ☐ von δ = bis

[¹³C] normal (δ = 220 bis 0) ☐

anderes Format:

Sonderwünsche: COSY ☐

¹³C — ¹H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☒ AM-300
☐ ARX-400
☐ DMX-600

gespeichert unter Nr. SIP 24.069/10

Bitte um Rücksprache ☐

Kommentar:

(Unterschrift)

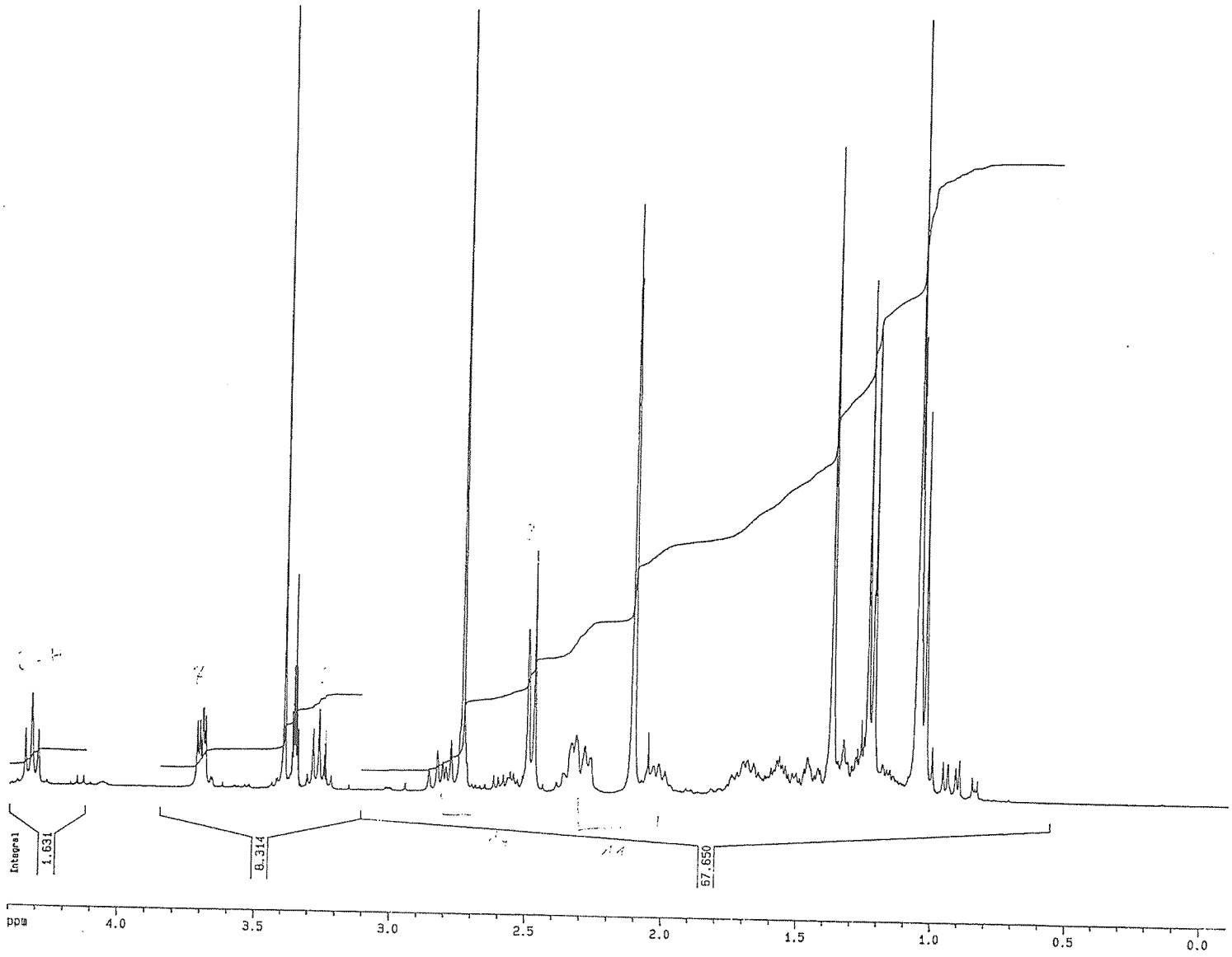
So 90.411 IRP_Fr. 24 IRP-② / KG

426.2

Evo C

17,6 mg

SIPZ4069 10 1 Pohlan



Current Data Parameters

NAME	SIPZ4069
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

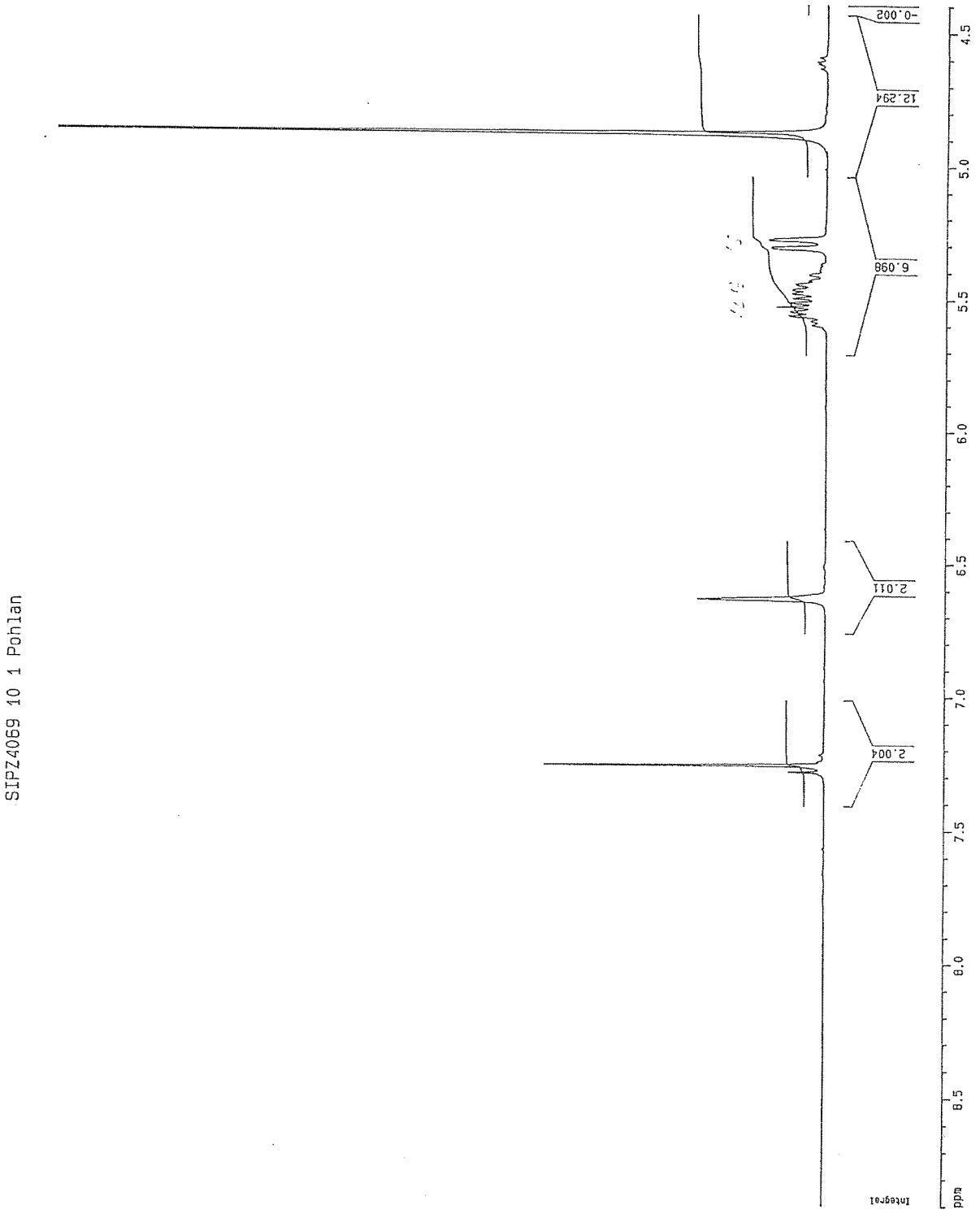
Date_	[redacted]
Time	9.18
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	64
DS	0
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	181
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	15.00 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-4.00 dB

F2 - Processing parameters

SI	16384
SF	300.1299927 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm


SIPZ4069 10 1 Pohlan
Integral
ppm
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8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
2.004
2.011
6.098
12.294
0.002

Einlieferungsdatum: [redacted]

Spektren-Nr.: 004174

273.

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

4-87

Substanz-Bez.: Epo C / So 90.426.2

Summenformel:

Substanzhersteller: Pohlan

Abteilung: NC (1.1.2) Tel.: 343

Kernart (¹H, ¹³C, ³¹P, andere?) — ¹H and ¹³C circled

Substanz-Menge: 5 mg, Molmasse:

geeignetes Lösungsmittel: DMSO-d6

weitere Messung nach Zugabe von

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒

im Tiefkühlfach ☐

im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen δ = 0 und 9

Gewünscht: nur Spektrum ☒

plus Integral ☒

Interpretation ☐

Zahl der Akkumulationen (falls > 104):

Art des Experiments

¹H Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en):

¹³C ¹H-Entkopplung:

Breitband ☒ selektiv ☐

DEPT ☒ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

Linienausdruck ☐

¹H

δ = 8.9 bis —0.1 (0.15 ppm/cm) ☒

11.9 bis —0.1 (0.2 ppm/cm) ☐

Drehungen:

10 Hz/cm ☐ von δ = bis

¹³C normal (δ = 220 bis 0) ☒

anderes Format:

Sonderwünsche: COSY ☒ ¹³C—¹H Korrel. Direkt ☒ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☐ AM-300 ☐ ARX-400 ☐ DMX-600

gespeichert unter Nr. S182, H179/10

1H 30 — COSY 1H

31 — direkt

32 — 13c

33 — Dept

Bitte um Rücksprache ☐

Kommentar:

(Unterschrift)

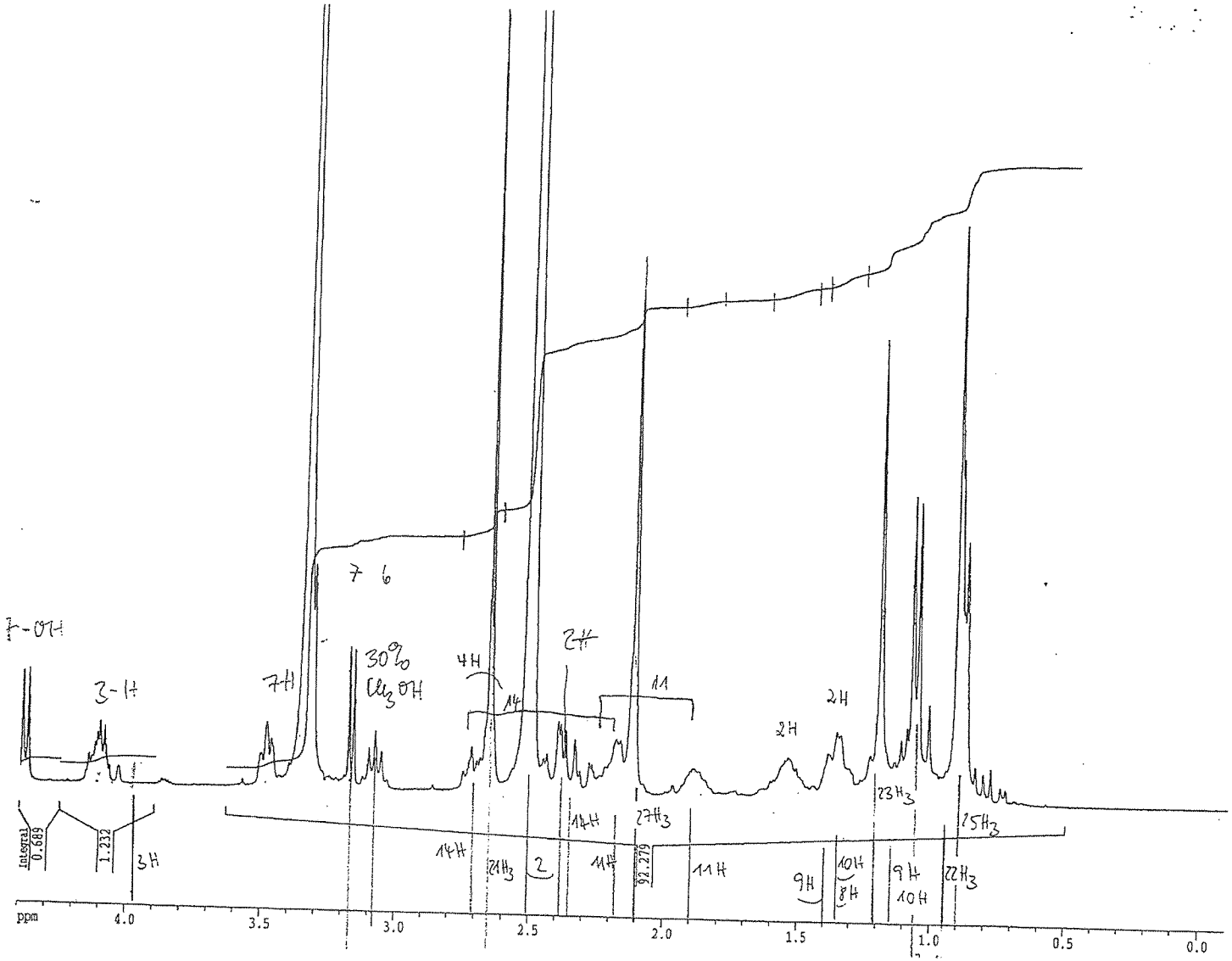
5090.426.2

Epothilon C

5-g

DMSO

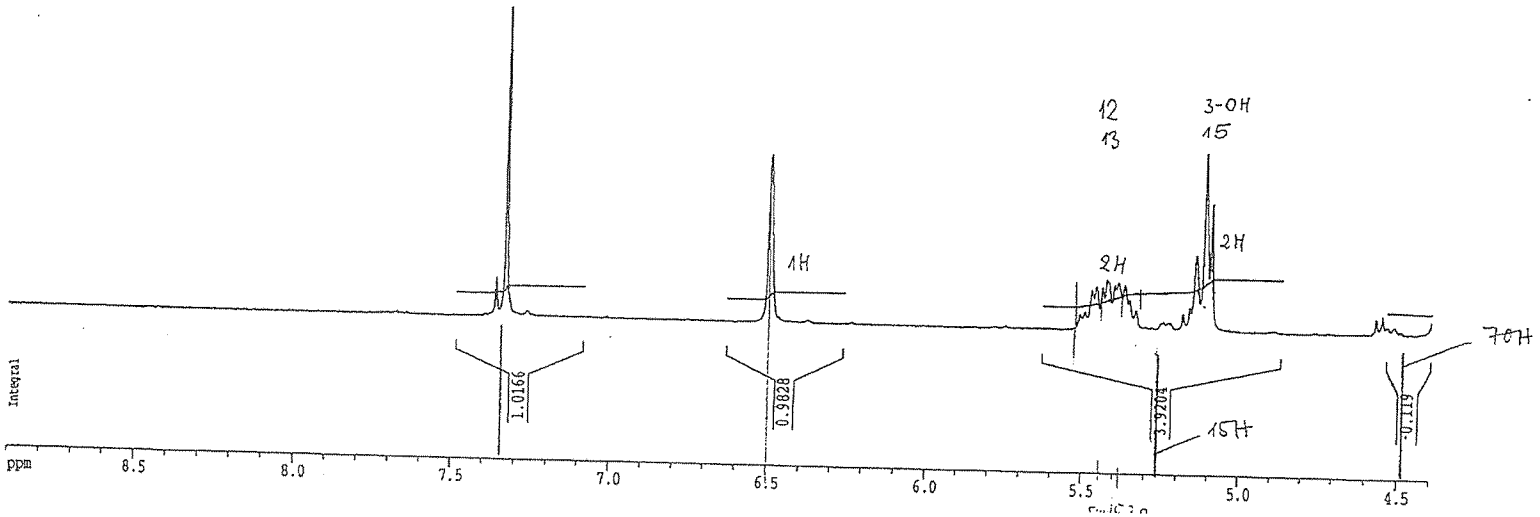
SIPZ4174 10 1



84-4

SIPZ4174 10 1

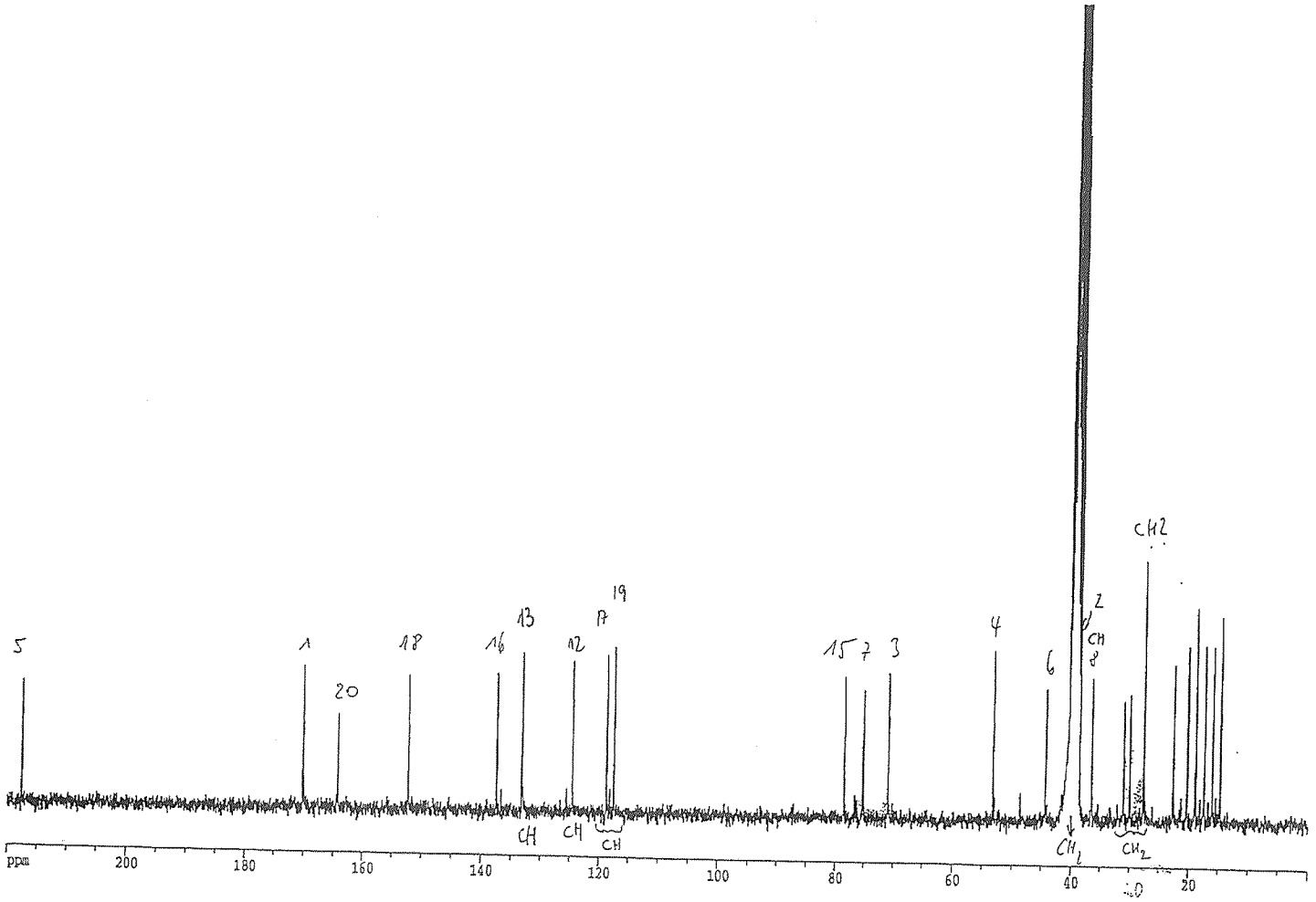
DMSO



EPO, C²

in DMSO

SIPZ4174 32 1



Current Data Parameters

NAME	SIPZ4174
EXPNO	32
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	3.47
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zgpg30
TD	32768
SOLVENT	DMSO
NS	16000
DS	2
SWH	23809.523 Hz
FIDRES	0.726609 Hz
AQ	0.6881780 sec
RG	1024
DW	21.000 usec
DE	5.50 usec
TE	300.0 K
D12	0.00002000 sec
PL13	25.00 dB
D1	1.00000000 sec
CPDPRG2	waltz16
PCPD2	97.00 usec
SFO2	300.1312005 MHz
NUC2	1H
PL2	-3.00 dB
PL12	12.20 dB
P1	11.00 usec
DE	5.50 usec
SFO1	75.4772501 MHz
NUC1	13C
PL1	-3.00 dB
D11	0.03000000 sec

F2 - Processing parameters

SI	32768
SF	75.4677834 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.40

1D NMR plot parameters

CX	30.00 cm
F1P	220.000 ppm
F1	16602.91 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	7.33333 ppm/cm
HZCM	553.43042 Hz/cm

4-90

/u/data/chk/nmr/SIPZ4174/32/pdata/1/screen Fri [redacted]:53:32 [redacted]

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#	ADDRESS	FREQUENCY [Hz]	[PPM]	INTENSITY	
1	6816.6	16416.988	217.5364	2.93	
2	11741.3	12838.661	170.1211	3.38	
3	12351.2	12395.488	164.2487	2.24	
4	13595.5	11491.410	152.2691	3.28	
5	15141.4	10368.112	137.3846	3.32	
6	15584.0	10046.493	133.1229	3.68	
7	16473.1	9400.500	124.5631	3.50	
8	17077.2	8961.576	118.7470	3.67	
9	17205.5	8868.332	117.5115	3.99	
10	21259.5	5922.664	78.4794	3.38	78.5
11	21602.4	5673.510	75.1779	3.04	75.2
12	22047.1	5350.393	70.8964	3.37	70.9
13	23883.2	4016.285	53.2185	3.94	
14	24807.3	3344.793	44.3208	3.11	
15	25221.4	3043.879	40.3335	22.30	
16	25250.1	3023.054	40.0575	58.88	
17	25278.9	3002.137	39.7804	108.85	
18	25307.6	2981.238	39.5034	119.63	
19	25336.4	2960.312	39.2262	97.81	
20	25365.2	2939.395	38.9490	47.04	
21	25394.1	2918.407	38.6709	15.56	
22	25613.2	2759.223	36.5616	3.35	
23	26182.3	2345.693	31.0820	2.84	
24	26292.8	2265.400	30.0181	2.99	
25	26542.5	2083.948	27.6137	6.08	9 + 11
26	27070.1	1700.635	22.5346	3.69	
27	27306.2	1529.078	20.2613	4.11	
28	27448.7	1425.549	18.8895	5.04	
29	27601.3	1314.677	17.4204	4.15	
30	27743.0	1211.706	16.0559	4.09	
31	27885.2	1108.357	14.6865	4.79	

EPO, C⁴

in DMSO

Einlieferungsdatum: [redacted]

Spektren-Nr.: 004045

145.

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

4-92

Substanz-Bez.: So 90.411 RP_Fl. 28 IRP-(1) (3)

Summenformel: _____

Substanzhersteller: Polyplan

Abteilung: NC (1.1.7) Tel.: 343

Kernart (¹H) (¹³C) ³¹P, andere?) _____

Substanz-Menge: 19,5 mg, Molmasse: _____

geeignetes Lösungsmittel: CD3OD weitere Messung nach Zugabe von _____

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒
im Tiefkühlfach ☐
im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen δ = 0 und 9

Gewünscht: nur Spektrum ☒
plus Integral ☒
Interpretation ☐

Zahl der Akkumulationen (falls > 104): _____

Art des Experiments

[¹H] Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en): _____

[¹³C] ¹H-Entkopplung:

Breitband ☒ selektiv ☐

DEPT ☒ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

[¹H]

δ = 8.9 bis —0.1 (0.15 ppm/cm) ☒

11.9 bis —0.1 (0.2 ppm/cm) ☐

Linienausdruck ☐

Drehungen:

10 Hz/cm ☐ von δ = _____ bis _____

[¹³C] normal (δ = 220 bis 0) ☒

anderes Format: _____

Sonderwünsche: COSY ☐

¹³C—¹H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☒ AM-300
☐ ARX-400
☐ DMX-600

gespeichert unter Nr. SP240545/10
_____, 120
_____, 121

Bitte um Rücksprache ☐

Kommentar:

_____

(Unterschrift)

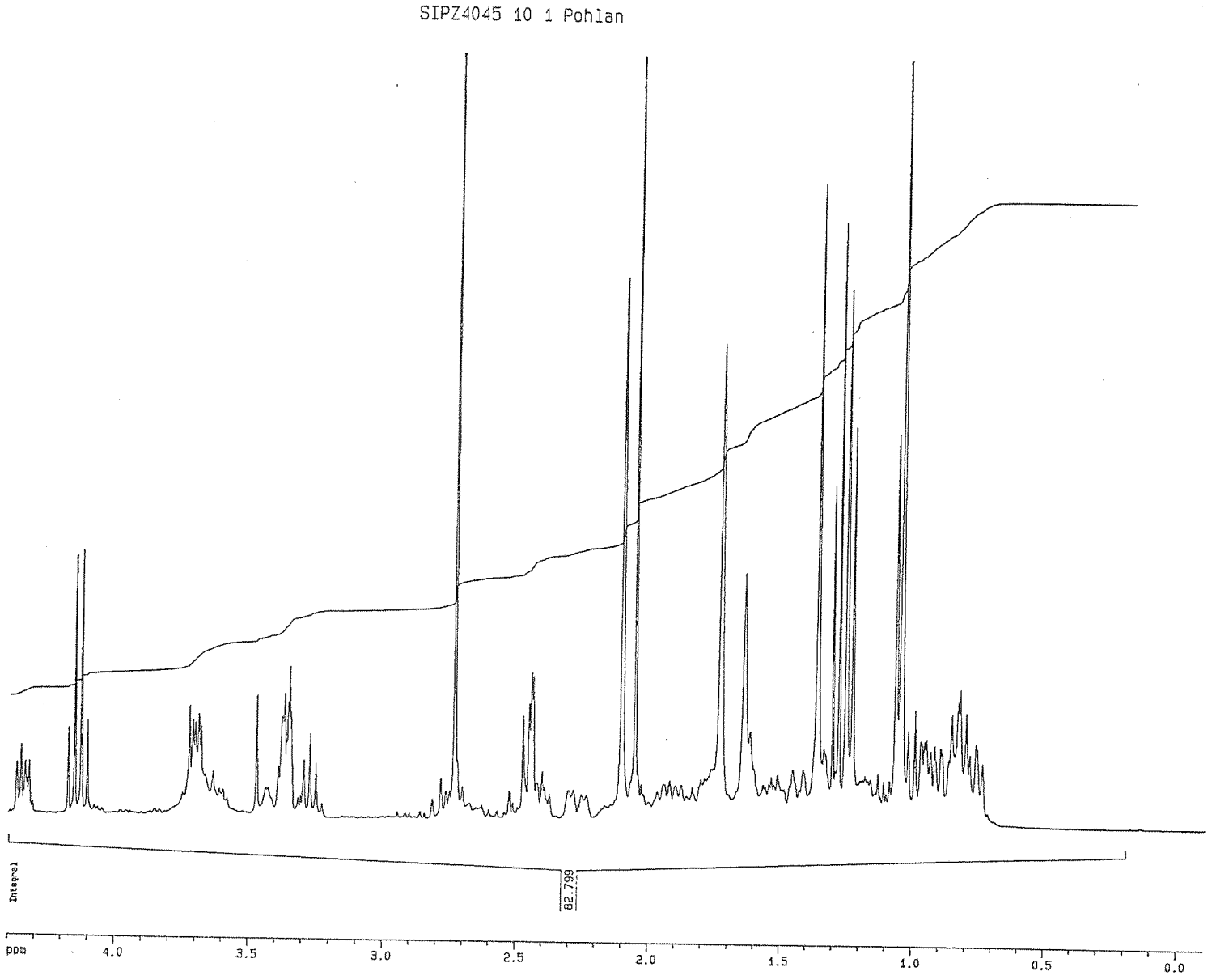
Evolution

So 90.411 IRP-Fl.28 IRP-(1)

428

19,5 mg → 31,2 mg

(1)



Current Data Parameters

NAME	SIPZ4045
EXPNO	10
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	10.13
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	88
DS	0
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	128
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	15.00 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-4.00 dB

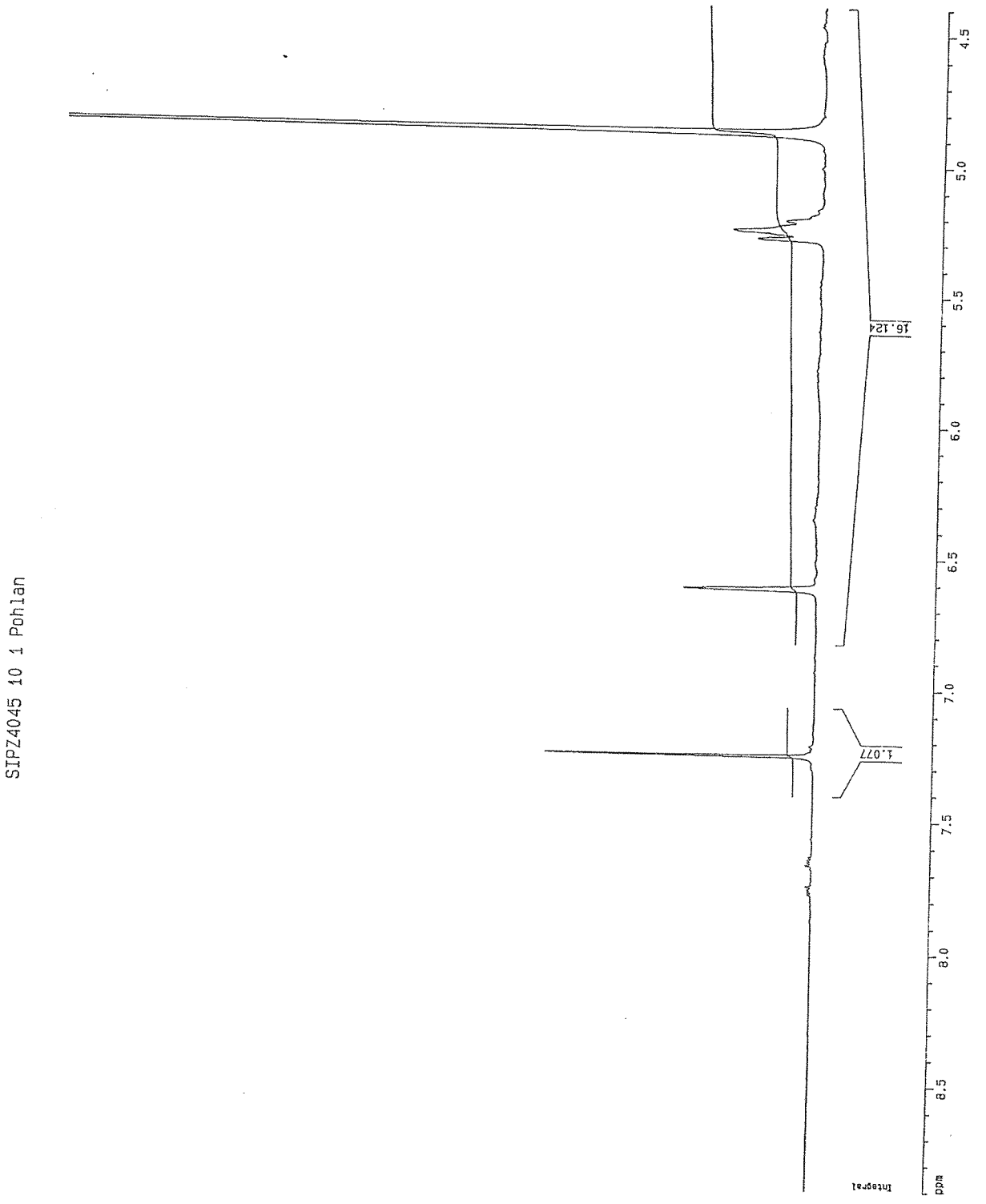
F2 - Processing parameters

SI	16384
SF	300.1299924 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm

S6-4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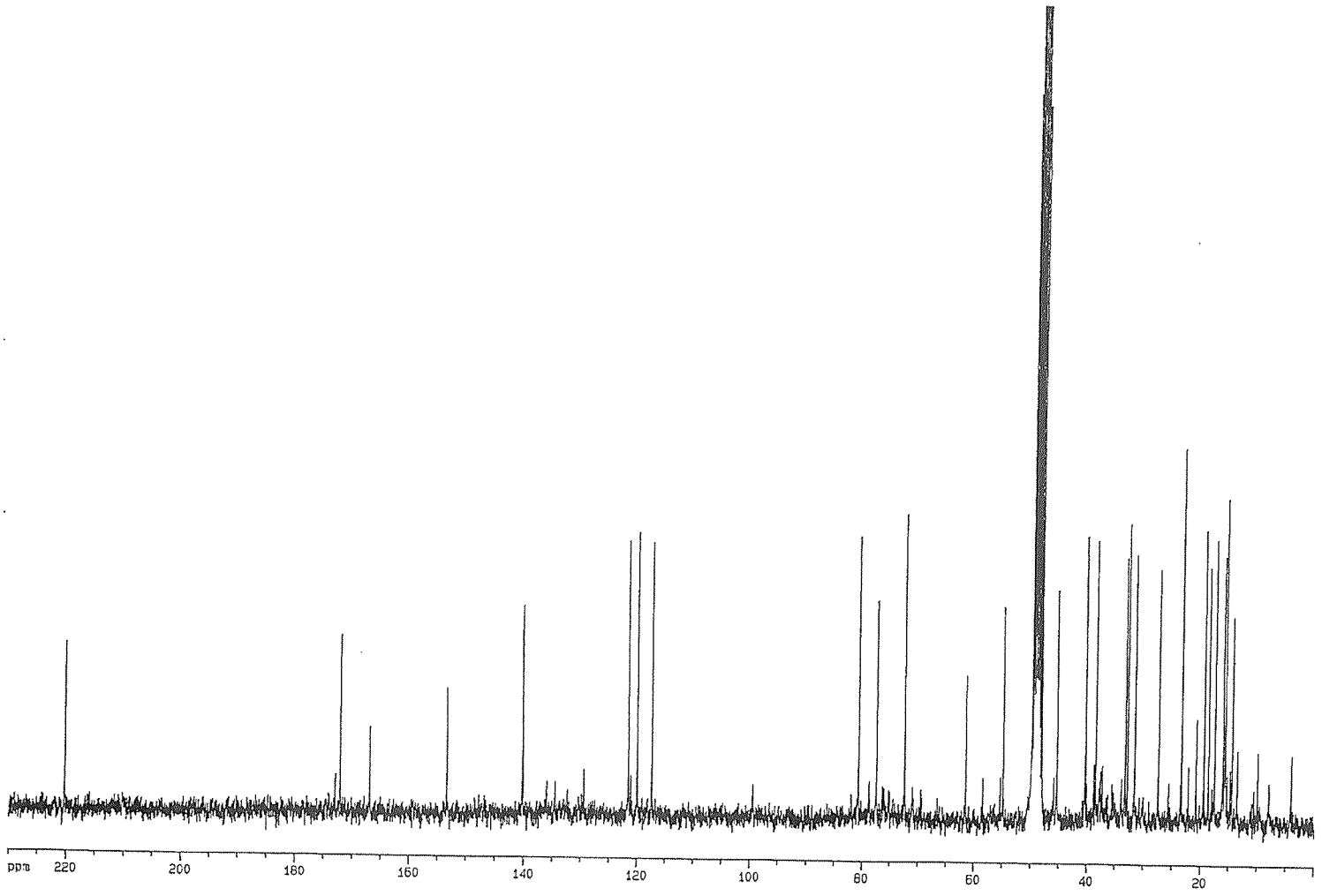
16.124
4.077
Integral
ppm
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5

SO90.411 RP_FI.28(RP-1)

428

19.5

SIPZ4045 20 1 Pohlan



Current Data Parameters

NAME	SIPZ4045
EXPNO	20
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	11.16
INSTRUM	spect
PROBHD	5 mm QNP 1H
PULPROG	zgpg30
TD	32768
SOLVENT	MeOH
NS	3760
DS	2
SWH	23809.523 Hz
FIDRES	0.726609 Hz
AQ	0.6881780 sec
RG	2048
DW	21.000 usec
DE	4.50 usec
TE	300.0 K
D12	0.00002000 sec
PL13	25.00 dB
D1	0.80000001 sec
CPDPRG2	waltz16
PCPD2	97.00 usec
SFO2	300.1312005 MHz
NUC2	1H
PL2	-3.00 dB
PL12	12.20 dB
P1	11.00 usec
DE	4.50 usec
SFO1	75.4772501 MHz
NUC1	13C
PL1	-3.00 dB
D11	0.03000000 sec

F2 - Processing parameters

SI	32768
SF	75.4676434 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	230.000 ppm
F1	17357.56 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	7.66667 ppm/cm
HZCM	578.58527 Hz/cm

4-45

/u/data/chk/nmr/SIPZ4045/20/pdata/1/screen Tue [redacted] 13:36:20 [redacted]

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F1=285.042ppm, F2=-30.451ppm, MI=0.00cm, MAXI=10000.00cm, PC=1.000

4-96

#	ADDRESS	FREQUENCY [Hz]	[PPM]	INTENSITY
1	6727.1	16623.523	220.2735	3.78
2	11639.8	13053.866	172.9730	0.87
3	11728.8	12989.188	172.1160	4.05
4	12268.2	12597.304	166.9232	1.96
5	13675.7	11574.554	153.3711	2.89
6	15038.5	10584.390	140.2507	4.37
7	15046.4	10578.604	140.1740	4.90
8	15482.8	10261.529	135.9726	0.76
9	15633.7	10151.841	134.5191	0.76
10	15860.8	9986.855	132.3329	0.57
11	16164.2	9766.376	129.4114	1.05
12	16979.5	9174.034	121.5625	6.44
13	17026.3	9139.974	121.1112	0.94
14	17142.8	9055.376	119.9902	6.49
15	17413.0	8859.035	117.3885	6.20
16	19273.8	7506.939	99.4723	0.74
17	21223.3	6090.396	80.7021	6.53
18	21426.0	5943.124	78.7506	0.84
19	21563.9	5842.914	77.4228	4.94
20	21672.9	5763.763	76.3740	0.73
21	21702.0	5742.604	76.0936	0.69
22	21782.6	5684.032	75.3175	0.66
23	22079.0	5468.641	72.4634	6.90
24	22226.1	5361.791	71.0475	0.71
25	22380.5	5249.614	69.5611	0.68
26	23214.8	4643.351	61.5277	3.28
27	23532.2	4412.754	58.4721	0.95
28	23849.3	4182.324	55.4188	0.98
29	23899.7	4145.718	54.9337	4.93
30	24427.1	3762.483	49.8556	16.56
31	24456.7	3741.021	49.5712	46.76
32	24486.1	3719.649	49.2880	92.59
33	24515.7	3698.168	49.0034	105.80
34	24545.1	3676.783	48.7200	92.28
35	24574.6	3655.310	48.4355	45.95
36	24604.1	3633.926	48.1521	16.24
37	24831.9	3468.352	45.9581	0.99
38	24886.7	3428.535	45.4305	5.26
39	25410.1	3048.276	40.3918	6.45
40	25435.5	3029.783	40.1468	0.86
41	25569.3	2932.605	38.8591	1.30
42	25601.9	2908.881	38.5447	6.36
43	25693.4	2842.427	37.6642	1.16
44	25729.8	2815.970	37.3136	1.27
45	25834.2	2740.097	36.3082	0.61
46	25910.7	2684.506	35.5716	0.85
47	25932.5	2668.660	35.3616	0.67
48	26078.1	2562.849	33.9596	0.97
49	26144.2	2514.872	33.3238	6.00
50	26188.5	2482.672	32.8972	6.95
51	26319.9	2387.194	31.6320	6.05
52	26484.0	2267.948	30.0519	0.56
53	26750.9	2074.054	27.4827	5.74
54	26942.3	1934.968	25.6397	0.88
55	27169.2	1770.080	23.4548	8.54
56	27172.5	1767.651	23.4226	7.38
57	27303.7	1672.336	22.1596	1.28
58	27438.6	1574.322	20.8609	2.43

59	27582.0	1470.167	19.4808	6.62
60	27666.0	1409.089	18.6714	5.77
61	27735.5	1358.635	18.0029	0.80
62	27784.2	1323.231	17.5338	6.44
63	27929.8	1217.439	16.1319	6.08
64	27959.4	1195.924	15.8468	2.67
65	27973.0	1186.054	15.7161	7.35
66	28015.2	1155.338	15.3090	1.07
67	28070.7	1115.056	14.7753	1.22
68	28102.7	1091.755	14.4665	4.71
69	28199.8	1021.210	13.5318	1.65
70	28503.1	800.822	10.6115	0.73
71	28571.0	751.523	9.9582	1.60
72	28776.3	602.322	7.9812	0.91
73	29178.2	310.304	4.1118	1.55

Einlieferungsdatum: [redacted]

004070
Spektren-Nr.: _____

168.

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

4-98

Substanz-Bez.: Jo 90.411/RP-Fl.28/RP-(1)/46-(1) =(1)

Summenformel: _____

Substanzhersteller: Pohlan

Abteilung: NC (1.1.7) Tel.: 343

Kernart (¹H, ¹³C, ³¹P, andere?) _____

Substanz-Menge: 11,2 mg, Molmasse: _____

geeignetes Lösungsmittel: (D₂O) weitere Messung nach Zugabe von _____

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern im Kühlschrank ☒
im Tiefkühlfach ☐
im Dunkeln ☐
Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen
δ = 0 und 9
Gewünscht: nur Spektrum ☒
plus Integral ☒
Interpretation ☐
Zahl der Akkumulationen (falls > 104): _____

Art des Experiments

[¹H] Standardspektrum ☒
Entkopplung ☐ Differenz-NOE ☐
Differenz-Entkopplung ☐
Entkoppler-Frequenz(en): _____

[¹³C] ¹H-Entkopplung:
Breitband ☐ selektiv ☐
DEPT ☐ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐
[¹H]
Linienausdruck ☐

δ = 8.9 bis —0.1 (0.15 ppm/cm) ☒
11.9 bis —0.1 (0.2 ppm/cm) ☐

Drehungen:
10 Hz/cm ☐ von δ = _____ bis _____

[¹³C] normal (δ = 220 bis 0) ☐
anderes Format: _____

Sonderwünsche: COSY ☐ ¹³C—¹H Korrel. Direkt ☐ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf
☒ AM-300
☐ ARX-400
☐ DMX-600

gespeichert unter Nr. SIR240700N0
_____, _____
_____, _____

Bitte um Rücksprache ☐

Kommentar:

_____
(Unterschrift)

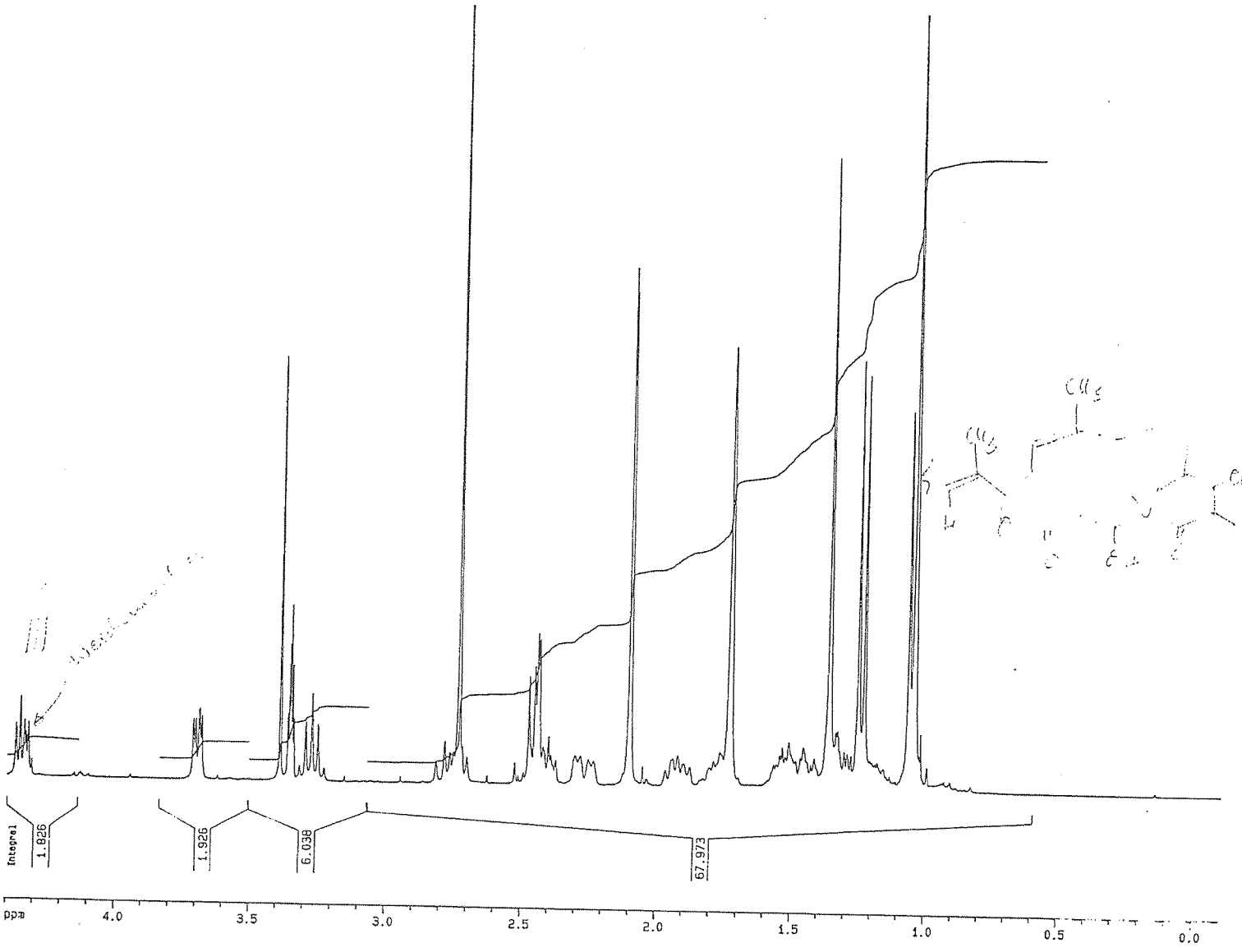
Eno D

So90.411/RP-Tr. 28/RP-① 1Kb ①

428.3

11,2mg

SIPZ4070 10 1 Pohlan



Current Data Parameters

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EXPNO	10
PROCNO	1

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PULPROG	zg30
TD	32768
SOLVENT	MeOH
NS	64
DS	0
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	181
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	15.00 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-4.00 dB

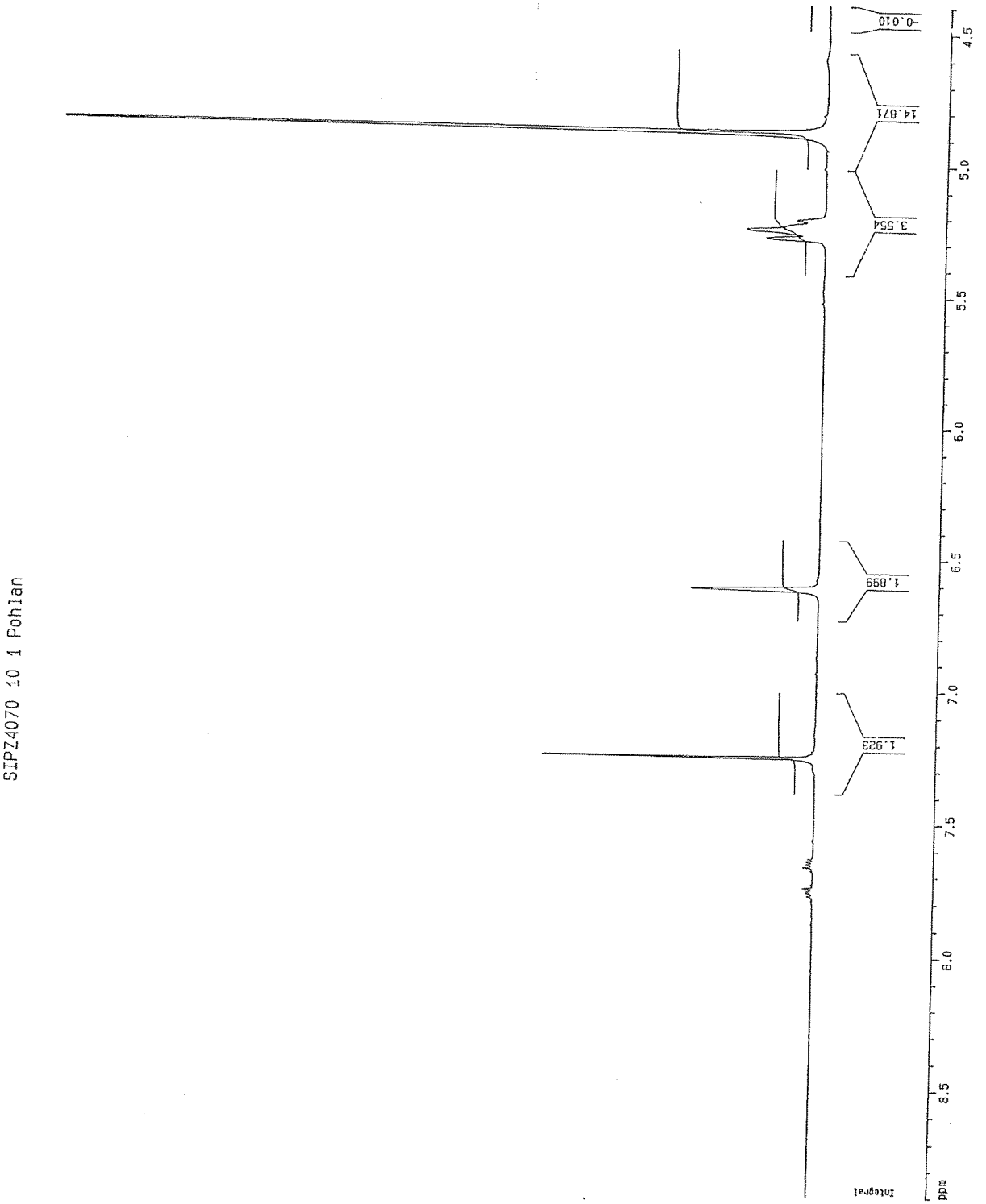
F2 - Processing parameters

SI	16384
SF	300.1299927 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm

f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-0.010
14.871
3.554
1.899
1.923
Integral
ppm
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5

Einlieferungsdatum: [redacted]

Spektren-Nr.: 004175

274.

NMR-ANTRAG

GBF — Abt. Molekulare Strukturforschung

4-101

Substanz-Bez.: Eko.D1 So90. 428.3

Summenformel: _____

Substanzhersteller: Roche

Abteilung: NC (1.1.2) Tel.: 363

Kernart (¹H, ¹³C, ³¹P, andere?) _____

Substanz-Menge: 11,2 mg, Molmasse: _____

geeignetes Lösungsmittel: DMSO-d6 weitere Messung nach Zugabe von _____

Substanz zurück: ja ☒ nein ☐

Strukturvorschlag:

Radioaktiv ☐ Toxisch ☐

Allgemeine Angaben

Probe lagern: im Kühlschrank ☒ im Tiefkühlfach ☐ im Dunkeln ☐

Probe auf Abruf beim Hersteller ☐

Signale erwartet zwischen δ = 0 und 9

Gewünscht: nur Spektrum ☒ plus Integral ☒ Interpretation ☐

Zahl der Akkumulationen (falls > 104): _____

Art des Experiments

[¹H] Standardspektrum ☒

Entkopplung ☐ Differenz-NOE ☐

Differenz-Entkopplung ☐

Entkoppler-Frequenz(en): _____

[¹³C] ¹H-Entkopplung: Breitband ☒ selektiv ☐ DEPT ☒ ohne ☐

Plot und Datenmanipulation

Gauss-Multiplikation ☐

[¹H]

δ = 8.9 bis −0.1 (0.15 ppm/cm) ☒

11.9 bis −0.1 (0.2 ppm/cm) ☐

Linienausdruck ☐

Drehungen: 10 Hz/cm ☐ von δ = _____ bis _____

[¹³C] normal (δ = 220 bis 0) ☒

anderes Format: _____

Sonderwünsche: COSY ☒ ¹³C—¹H Korrel. Direkt ☒ Long-range ☐

(Nicht vom Antragsteller auszufüllen)

gemessen auf ☐ AM-300 ☐ ARX-400 ☐ DMX-600

gespeichert unter Nr. S1R2,4175/10 1H ✓ S1R8,4175/10 11 COSY ✓ 12 direkt 20 LR ✓ 21 Dept ✓ 35 1H 36 dito

Bitte um Rücksprache ☐

Kommentar:

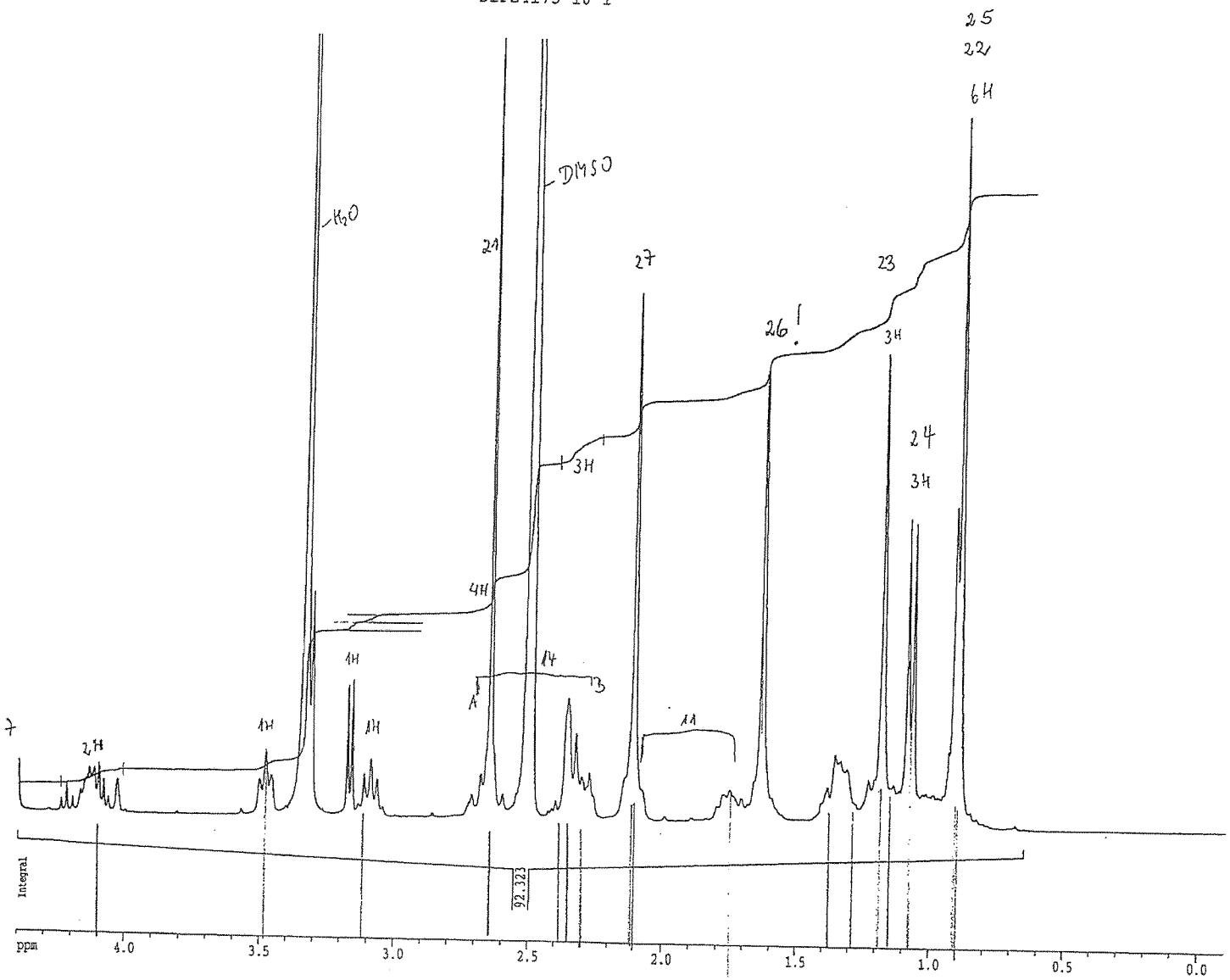
(Unterschrift)

So 90.428.3

Epothilon D

11,2-g

SIPZ4175 10 1



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EXPNO	10
PROCNO	1

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PULPROG	zg30
TD	32768
SOLVENT	DMSO
NS	104
DS	2
SWH	6172.839 Hz
FIDRES	0.188380 Hz
AQ	2.6542580 sec
RG	181
DW	81.000 usec
DE	4.50 usec
TE	300.0 K
D1	1.00000000 sec
P1	15.00 usec
DE	4.50 usec
SFO1	300.1318534 MHz
NUC1	1H
PL1	-4.00 dB

F2 - Processing parameters

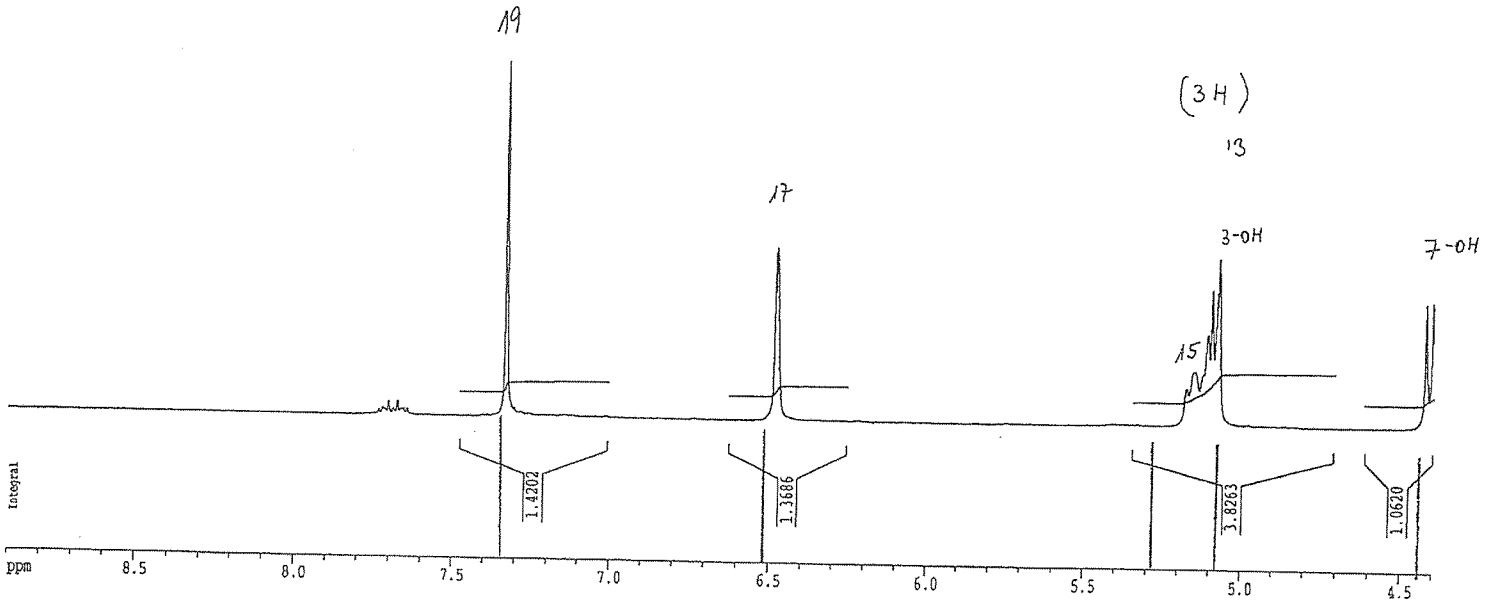
SI	16384
SP	300.1300011 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	4.400 ppm
F1	1320.57 Hz
F2P	-0.100 ppm
F2	-30.01 Hz
PPMCM	0.15000 ppm/cm
HZCM	45.01950 Hz/cm

20 V - 4

SIPZ4175 10 1

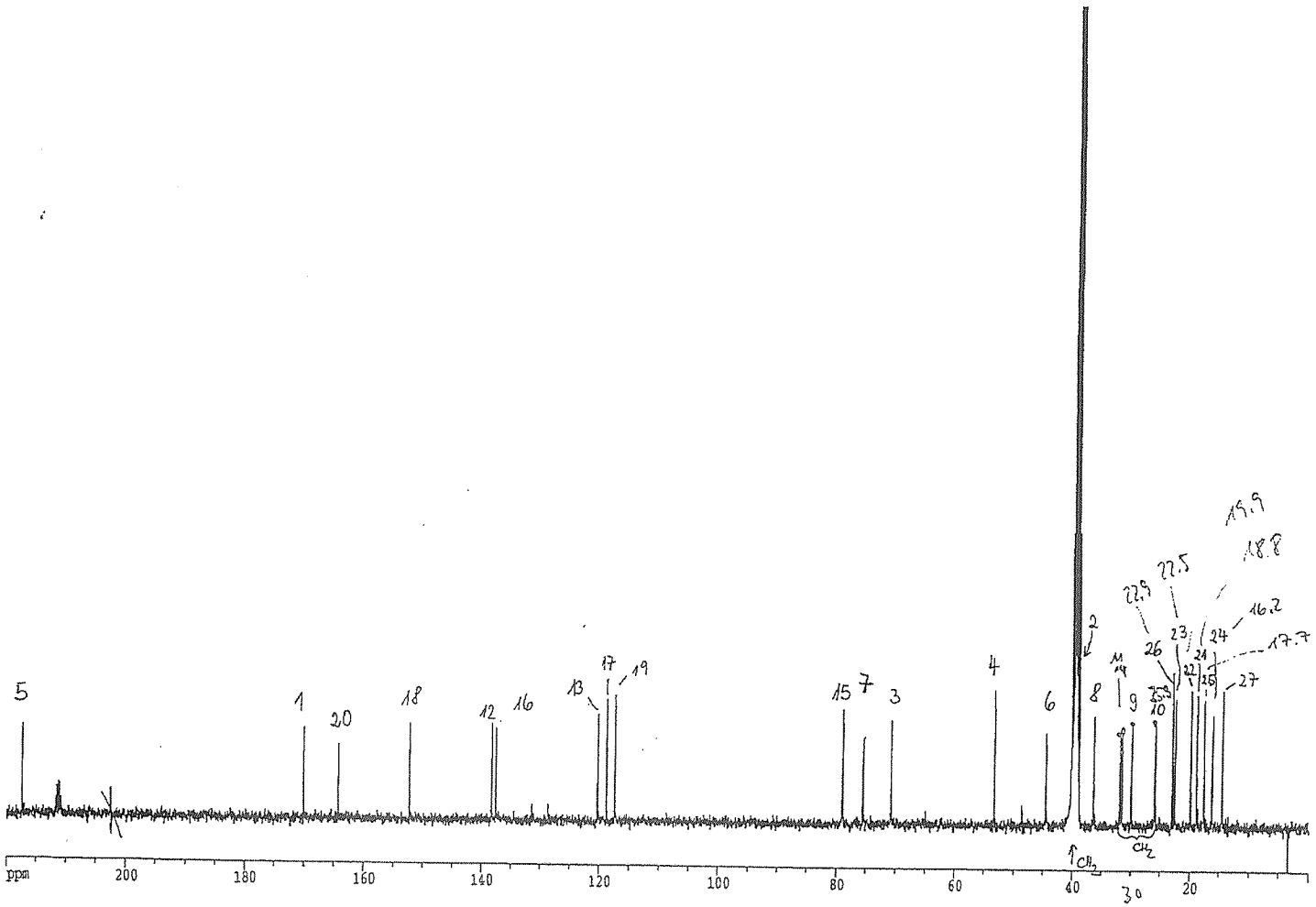


M

4-103

EPO "D"

SIPR4175 20 1



Current Data Parameters

NAME	SIPR4175
EXPNO	20
PROCNO	1

F2 - Acquisition Parameters

Date_	[redacted]
Time	10.47
INSTRUM	arx400
PROBHD	5 mm QNP 1H
PULPROG	zgpg30
TD	32768
SOLVENT	DMSO
NS	2800
DS	2
SWH	33333.332 Hz
FIDRES	1.017253 Hz
AQ	0.4915700 sec
RG	16384
DW	15.000 usec
DE	21.43 usec
TE	300.0 K
D12	0.00002000 sec
DL6	22.00 dB
D1	1.00000000 sec
CPDPRG	waltz16
P31	75.00 usec
D11	0.03000000 sec
DL5	19.00 dB
P1	10.00 usec
DE	21.43 usec
SFO1	100.6254358 MHz
NUCLEUS	13C

F2 - Processing parameters

SI	32768
SF	100.6128210 MHz
WDW	EM
SSB	0
LB	2.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	30.00 cm
F1P	220.000 ppm
F1	22134.82 Hz
F2P	0.000 ppm
F2	0.00 Hz
PPMCM	7.33333 ppm/cm
HZCM	737.82739 Hz/cm

4-404

EPO „D"

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4-105

#	ADDRESS	FREQUENCY [Hz]	[PPM]	INTENSITY	
1	7281.0	21874.760	217.4152 – 5	2.07	
2	7869.0	21276.707	211.4711	0.67	
3	7890.0	21255.285	211.2582	0.75	
4	7910.4	21234.568	211.0523	0.74	
5	8761.3	20368.973	202.4491	0.63	
6	11965.3	17109.715	170.0550 – 1	2.13	
7	12546.3	16518.658	164.1805 – 20	1.74	
8	13727.1	15317.464	152.2417 – 18	2.20	
9	15100.4	13920.527	138.3574 – 13	2.29	
10	15178.4	13841.204	137.5690 – 16	2.18	
11	16887.6	12102.428	120.2871 – 12	2.50	
12	17037.1	11950.432	118.7764 – 17	2.78	
13	17170.7	11814.513	117.4255 – 19	2.95	
14	20978.3	7941.236	78.9287	2.64	
15	21325.2	7588.323	75.4210 – 7	1.94	
16	21783.3	7122.308	70.7893 – 3	2.42	
17	23516.1	5359.594	53.2695 – 4	3.09	
18	23983.1	4884.557	48.5481	0.46	
19	24390.8	4469.850	44.4262 – 6	2.11	
20	24816.1	4037.134	40.1254	6.66	
21	24836.8	4016.094	39.9163	18.85	+
22	24857.5	3995.087	39.7075	36.05	+
23	24878.1	3974.093	39.4989	42.10	
24	24898.8	3953.056	39.2898	35.53	
25	24919.4	3932.073	39.0 39.0812 – 2	17.61	
26	24939.8	3911.324	38.8750	6.66	
27	25192.9	3653.922	36.3167 – 8	2.51	
28	25633.0	3206.134	31.8661 – 11	2.02	* kommen vielleicht rein
29	25662.2	3176.465	31.5712 – 14	2.13	*
30	25827.4	3008.435	29.9011 – 9?	2.36	
31	26223.3	2605.679	25.8981 – 10?	2.41	
32	26515.7	2308.261	22.9420 – 26	3.60	
33	26556.8	2266.417	22.5261 – 23	2.95	
34	26819.3	1999.370	19.8719 – 22	3.18	
35	26921.3	1895.664	18.8412 – 21	3.07	
36	26945.8	1870.694	~~18.5930~~	0.43	
37	27036.9	1778.058	17.6723 – 25	2.90	
38	27182.0	1630.464	16.2053 – 24	2.54	
39	27348.0	1461.631	14.5273 – 27	3.11	

Reply to the

Opposition Statement against EP-B-1186606

concerning the identity of epothilones C and D

by Gerhard Höfle

GBF, September 8, 2005

Contributions by Dr. K. Gerth, H. Steinmetz (GBF),

Prof. D. Schinzer (University of Magdeburg)

Introduction

In 1990/1991 epothilones A and B have been produced for the first time on the gram-scale with *Sorangium cellulosum* So ce90 wild strain. A patent was filed for epothilones A and B November 19, 1991, and the strain was deposited at the German Strain Collection DSMZ under the code DSM 6773. At that time during large scale isolation work more lipophilic epothilones were observed during RP chromatography of epothilones A and B[1)] which however were not isolated because of the small amounts present and, later lack of interest in epothilones.

After the tubulin activity was published by Bollag et al.in 1995 work was resumed, and a number of other *Sorangium cellulosum* strains were identified to produce epothilones A and B. From these strain So ce1198 was selected for further work because of low abundance of other unwanted metabolites which otherwise interfere with isolation. As side products from this strain epothilones C and D were isolated in pure state and the structures elucidated in June 1996 as documented already. In Sptember/October a 350 L fermenter with strain So ce1198 was run for the production of epothilones A and B. From this as side products several hundred milligrams of epothilones C and D were isolated. With this material a complete set of NMR spectra in DMSO-D_6 was obtained, and the chromatographic behavior determined as basis for the patent application November 18, 1996 which represents the first description of epothilones C and D. Later epothilones C and D were obtained by total synthesis by the Danishefsky, Nicolaou and Schinzer groups. Interestingly, in papers by Danishefsky et al. they were named desoxyepothilones A and B[2)]. From natural sources epothilones C and D were re-isolated using So ce90/B2, a mutant with improved epothilone A and B production (Hardt et al.[3)]), and P450 knock-out mutants obtained by UV-irradiation (Gerth et al.[4)]) or genetic engineering (Lau et al[5)]). To demonstrate feasability of epothilone A and B total synthesis via C and D, epoxidation using dimethyl dioxirane and *m*-chloroperbenzoic acid were performed in June 1996 and in more detail in November/ December 1996 (Höfle et al.[6)]).

In the present Opposition Statement of Sloan Kettering Institute for Cancer Research it is claimed that the compounds described in the GBF patent EP-B-1186606 (7) are not epothilones C and D but rather other epothilones of non-defined structure. This conclusion is based on two observations:

1. Certain signals in the proton and carbon NMR spectra taken from MSKCC epothilones C and D differ significantly from those given in EP-B-1186606 (Prof. J. D. Roberts),

2. Attempts to obtain epothilones C and D by cultivation of *Sorangium cellulosum* strain So ce90 obtained as DSM6773 from the DSMZ failed (Dr. P. J. Licari, KOSAN).

In the following it is clearly proven that the compounds isolated in 1996 had the structures claimed in EP-B-1186606, today known as epothilones C and D.

It is further demonstrated that by re-fermentation of strain DSM6773 and isolation as described indeed epothilones C and D are obtained.

Prof. Schinzer confirmes that epotilones C and D obtained in 1996 from GBF were identical with his synthetic compounds.

(1) EP 07 127 252 9

(2) So ce 90 = DSM 6773

NMR spectroscopy of epothilones C and D

From the first proton NMR spectra recorded in June 1996 (as documented before) and biosynthetic considerations the structures of epothilones C and D were unequivocally derived. When larger amounts of the compounds became available in Oct./Nov. complete sets of 1D and 2D spectra were recorded in DMSO-D_6. The particular solvent was chosen to allow observation of hydroxy proton signals and couplings, and to facilitate comparison with the published data for epothilones A and B (Höfle et al.[7]). In the appendix NMR Request Forms, shift records and 1D proton and carbon spectra of epothilones C and D are given. In Tab. 1 and 2 carbon shifts, in Tab. 3 and 4 proton shifts determined November 14 and 15, 1996, are compared with those from recent samples measured May 18, 2005 at GBF, and KOSAN (Opposition Statement, p. 54-60).

^{13}C shifts for epothilone C (Tab. 1)
The shifts for all carbons except C1 and C2 are found within +/- 0.1 ppm. C2 deviates by 0.2 ppm due to partial overlap with solvent signals, whereas carbonyl C1 is by 0.5 ppm too high in the KOSAN spectrum. This may be attributed to a solvent induced shift, which is common with carbonyl carbons.

^{13}C shifts for epothilone D (Tab. 2)
The shifts for all carbons except C2 are in excellent agreement within +/- 0.1 ppm. C2 deviates by 0.3-0.4 ppm due to overlap with solvent signals. The values reported by KOSAN are consistantly to high by 0.2 ppm which is attributed to an offset of the reference.

^{1}H shifts for epothilone C (Tab. 3)
In general most proton NMR signals of complex natural products are complex because of multiple couplings and signal overlap. Under these circumstances the shift differences of +/- 0.03 ppm between GBF, Nov. 96 and KOSAN measurements indicate excellent Übereinstimmung.

^{1}H shifts for epothilone D (Tab. 4)
The majority of shifts are identical for GBF, Nov. 96 and KOSAN measurements, and only few deviate up to +/- 0.3 ppm.

The above comparison of chemical shift data unequivocally prove that the epothilones isolated in 1996 were indeed epothilones C and D.

How can the deviating values in the table of EP-B-1186606 (iii) be explained although they were extracted from the spectra measured November 14/15, 1996 which contain the correct ones ?

As basis for writing the table for the patent application Mr. Steinmetz used an existing table with the data for epothilones A and B and replaced the values atom for atom with the coresponding values from the epothilone C and D spactra. Obviously, he started with the signals around the 12,13 double bond and epoxide, respectively, and others differing significantly in the olefin and epoxide series. These values are marked in blue in Tab. 1-4. Later, he apparently forgot to adjust also the slightly deviating values. They are marked in red like those for epothilones A and B in Tab. 1-4. When I checked the table fabricated by Mr. Steinmetz for plausibility before submission of the patent application I had no chance to discover the small differences.

(iii) aber Bios DE 195 42 986 17.11.1995

Coming back to the claim on p. 39 of the Oppsition Statement that the compounds described in the patent were actually isomers of epothilones C and D with the same molecular mass, viz. m/z 477 and 491, respectively. This can be ruled out by comparison of the ^{13}C shifts for e.g. the known epothilone C isomers, 12*E*-epothilone C, epothilones D1 and D2. As shown in Tab. 5 shift differences of 2 up to 6.6 ppm are observed which is by far above the (slightly wrong) values in the patent.
Even though the values given in EP-B-1186606 deviate slightly more than is generally observed as experimental error, they are not misleading in a structural assignment. To demonstrate the variability of chemical shifts for complex natural products published data for epothilone C in $CDCl_3$ from different authors are summarized in Tab. 6.

Production of epothilones C and D *with Sorangium cellulosum* DSM 6773

Epothilones C and D are the primary products of epothilone biosynthesis. After release from the polyketide synthase complex they are modified by so-called "decorating enzymes" to the epoxides, epothilone A and B[4,8,9], and then to the 21-hydroxy derivatives, epothilones E and F[10].
Thus any *Sorangium* strain capable of epothilone A and/or B synthesis has to produce as intermediates epothilones C and/or D. (Molnar et al.[8] and Tang et al.[9]). Whether these intermediates can be observed and isolated from a culture depends on a variety of preconditions which are not well defined and mostly unknown. Certainly the harvest time, media composition, export activity of the organism and presence of XAD adorber resin are essential factors. It is not surprising for the expert that in a single run these compounds may be missed as they are minor side products with wild strains or mutants generated for production of epothilones A and B. Only P450 knock-out mutants produce relaibly high amounts of epothilones C and D (Gerth et al.[4], Lau et al.[5]).

KOSAN ordered strain DSM6673 from DSMZ three times, November 26, 1999, March 9, 2000 and May 18, 2004. From this and data in the Opposition Statement (Appendix 4, p.1-2) it follows that the second shipment of March 9, 2000 was used for the attempt to reproduce the production of epothilones C and D. No information is given whether and how the strain was preserved or kept in culture for several years until the experiments were performed in August-November 2004. It is well known that myxobacteria like other microorganisms change their properties during extended cultivation due to clonal selection or unfavourable conditions for preservation. Thus without an analytical check for epothilone production on the shake flask level it was high-risk to start with a 70 L batch. Even though the procedure in the patent could be reproduced yielding 167 g of crude extract (180 g in the patent). This material was separated on LH-20 under supposedly the same conditions as described in the patent. The fraction eluting between 240-300 minutes was collected without checking the presence of epothilones by TLC or HPLC. The fraction contained only 35 mg instead of 72 g in the patent. From the fact that epothilones A, B, C and D co-elute from Sephadex LH-20 it must be concluded at this point that the right fraction was missed. This is however not too surprising, as it is common experience that retention times are very sensitiv to a number of parameters which, particularly in large scale chromatography, cannot be controled. The expert in the field in such case certainly would have checked adjoining fractions for the presence of epothilones before discarding them and not contined tedious work on a tiny fraction (2,250-times less than expected). In addition, this fraction was not analysed for the presence of epothilones C and D but stupidly processed further without a result. Obviously, this was the actual purpose of the exercise.

It is important to notice at this point that KOSAN reported the isolation of epothilone C and D from a not specified *Sorangium cellulosum* (wild)strain: "They are secreted as minor products during the fermentation process with a combined yield of about 0.4 mg/L" (Lau et al.[5]).

To demonstrate that wild strain DSM 6673 indeed produces epothilones C and D it was newly ordered from DSMZ, and obtained May 24, 2005. The culture (now coded as So ce90wild DSM 6773) on slant agar was propagated on agar plates and taken into liquid culture as described by Gerth et al.[11] and in epothilone A/B patents.[12(a)]

In detail,

1. agar plates with probion medium[13] were inoculated on May 24, and propagated,
2. H medium[12] plus 1.2% HEPES buffer (500 mL) was inoculated on June 16, and the culture propagated
3. 22 shaking flasks with H medium[12] plus 1.2% HEPES buffer (550 mL each) were inoculated on July 26,
4. a 150 L fermentor with H medium[12] (100 L) and 2 Kg of wet XAD-16 adsorber was inoculated with 10 l of the above culture on August 1 (pH adjustment with 10% aq. acetic acid, and 10% aq. KOH, 32°C, 30% oxygen saturation, see also Figure 1),
5. the adsorber resin was harvested by sieving on August 15 and immediately processed further as described below.

When the production of epothilones C and D was determined on the shake flask level a constantly high proportion of spirangiens was observed and only very little of epothilones A-D. This unfavourable production profile may be due to the short time of adaption of the strain to the liquid medium. It was later reproduced with the production fermentor containing 2.4 g of spirangiens A and C, and only 3.1 mg of epothilone A, 1.8 mg of epothilone B, 1.4 mg of epothilone C, and 0.5 mg of epothilone D (Figure 3 - 7). To facilitate the isolation of such small amounts of epothilones in presence of co-eluting spirangiens an additional extraction step with sodium carbonate solution was introduced which removed most of the spirangiens as carboxylic acid salts.

The entire isolation process from wet XAD adsorber resin to pure epothilones C and D is given in Figures 2a and 2b. It should be noted that the presence of epothilones in LH20 and RP-silica gel chromatography fractions was monitored by HPLC/MS. Thus no loss of material occurred, and the expected amounts of 1.4 mg of epothilone C and 0.5 mg of epothilone D were obtained in pure state. From physical data, in particular proton and carbon NMR spectra, the identity of the compounds is equivocally proven (Table 7).

Thus, So ce90wild DSM 6773 (patent strain of DE 4138042) is indeed producing Epothilones C and D.

(a) DE 4138 042

Statement from Prof. Schinzer

In 1996 Dieter Schinzer was Professor for Organic Chemistry at the University of Braunschweig and a colleage of mine. Like other synthetic chemists he obtained the absolute configuration of epothilone A and B around November 1995. He developed plans for a total synthesis and discussed certain crucial steps with me. In summer 1996 I mentioned to him the isolation of epothilones A and B and my preliminary experiments on the epoxidation to give preferably the desired stereoisomer. In October he received samples of ca. 5 mg each for comparison purposes. Both were found to be identical with his compounds from total synthesis. This was acknowledged for epothilone C in a paper on epothilone A total synthesis.[14]
From my recent contacts with Prof. Schinzer I know that he is willing to witness this.

References

1) H. Steinmetz, unpublished.
2) D.-S. Su et al. *Angew. Chem. Int. Ed.*, *36*, 757, **1997**.
3) I. H. Hardt et al., J. Nat. Prod. 2001, *64*, 847.
4) Gerth, K. et al., *J. Antibiot.*, *54*, 144, **2001**.
5) J. Lau et al., Biotech & Bioengin. *78*, 280, **2002**.
6) G. Höfle et al. Pure Appl. Chem. *71*, 41, **2002**.
7) Höfle, G. et al *Angew. Chem. Int. Ed.*, *35*, 1567, **1996**.
8) I. Molnar et al. Chemistry & Biology *7*, 97, **2000**.
9) L. Tang et al., *Science*, **2000**, 287, 640.
10) Gerth, K. et al., *J. Antibiot.*, *55*, 41, **2002**.
11) Gerth, K. et al., *J. Antibiot.*, *49*, 560, **1996**.
12) H medium is the production medium used in DE 4138042 (Nov. 19,1991).
13) Pradella et al. *Arch Microbiol, 178*, 484, **2002**.
14) D. Schinzer et al., Chem. Eur. J. *5*, 2483, **1999**

Tab. 1 ^{13}C-NMR chemical shifts of epothilone C in DMSO-D_6

C-Atom	Epo A[1]	EP-B-1186606 18.11.96	GBF[3] 15.11.96	GBF[4] 18.5.05	Kosan[5]
1	170.3	170.3	170.1	170.0	170.6
2	38.4	38.4	38.7	38.9	38.8
3	71.2	71.2	70.9	70.8	70.8
4	53.1	53.1	53.2	53.2	53.2
5	217.1	217.1	217.5	217.5	217.5
6	45.4	45.4	44.3	44.2	44.3
7	75.9	75.9	75.2	75.1	75.1
8	35.4	35.4	36.6	36.6	36.6
9	29.6	27.6	27.6	27.5	27.6
10	23.6	30.0	30.0	30.0	30.0
11	27.2	27.6	27.6	27.6	27.6
12	56.6	133.1[2]	133.1	133.0	133.1
13	54.4	124.6[2]	124.6	124.6	124.5
14	32.1	31.1	31.1	31.1	31.1
15	76.3	76.3	78.5	78.4	78.4
16	137.3	137.3	137.4	137.4	137.3
17	119.1	119.1	118.7	118.7	118.7
18	152.1	152.1	152.3	152.2	152.3
19	117.7	117.7	117.5	117.4	117.5
20	164.2	164.2	164.2	164.2	164.2
21	18.8	18.8	18.9	18.8	18.9
22	20.8	20.8	20.3	20.1	20.2
23	22.6	22.6	22.5	22.4	22.5
24	16.7	16.7	16.1	15.9	16.0
25	18.4	18.4	17.4	17.3	17.5
26	-	-	-	-	-
27	14.2	14.2	14.7	14.7	14.7

Refences and comments:

1. G. Höfle et al. Angew. Chem. Int. Ed. Engl. 1996, 35, 1567-1569.
2. Misalignment corrected.
3. Spectrum taken Nov. 15, 1969 from a sample of epothilone C isolated Oct./Nov. 1996.
4. Recent sample of epothilone C.
5. Opposition Statement, p. 55-56.

Conclusion:

Chemical shifts for C1-C8 and C15-C27 in EP-B-1186606 are identical with epothilone A (red), those for C9-C14 are identical with epothilone C(blue).

Tab. 2 ^{13}C-NMR chemical shifts of epothilone D in DMSO-D_6

C-Atom	Epo B[1]	EP-B-1186606 18.11.96	GBF[2] 14.11.96	GBF[3] 17.5.05	Kosan[4]
1	170.1	170.1	170.1	170.1	170.3
2	38.2	39.0	39.0	38.7	39.1
3	70.0	70.8	70.8	70.8	71.0
4	53.2	53.2	53.3	53.3	53.5
5	217.4	217.4	217.4	217.5	217.6
6	44.9	44.4	44.4	44.4	44.7
7	75.5	75.5	75.4	75.4	75.6
8	35.6	36.3	36.3	36.3	36.5
9	29.6	29.9	29.9	29.9	30.1
10	23.0	25.9	25.9	25.9	26.1
11	32.1	31.6	31.6	31.6	31.8
12	61.0	138.3	138.4	138.4	138.6
13	61.5	120.3	120.3	120.3	120.5
14	33.0	31.9	31.9	31.9	32.1
15	76.6	76.6	78.9	79.0	79.1
16	137.2	137.2	137.6	137.6	137.8
17	119.2	119.2	118.8	118.8	119.0
18	152.1	152.1	152.2	152.3	152.5
19	117.7	117.7	117.4	117.4	117.7
20	164.3	164.3	164.2	164.2	164.4
21	18.9	18.9	18.8	18.9	19.1
22	19.7	19.7	19.9	19.9	20.1
23	22.5	22.5	22.5	22.6	22.7
24	16.4	16.4	16.1	16.2	16.4
25	18.4	18.4	17.7	17.7	17.9
26	22.1	22.9	22.9	23.0	23.2
27	14.1	14.1	14.5	14.6	14.7

Refences and comments:

1. G. Höfle et al. Angew. Chem. Int. Ed. Engl. 1996, 35, 1567-1569.
2. Spectrum taken Nov. 14, 1969 from a sample of epothilone D isolated Oct./Nov. 1996.
3. Recent sample of epothilone D.
4. Opposition Statement, p. 59-60.

Conclusion:

Chemical shifts for C7, C15-C25, and C27 in EP-B-1186606 are identical with epothilone B(red), those for C1-C6, C8-C14, and C26 are identical with epothilone D (blue).

Tab. 3 ^{1}H-NMR chemical shifts of epothilone C in DMSO-D_6

H-Atoms	Epo A[1]	EP-B-1186606 18.11.96	GBF[2] 15.11.96	Kosan[3]
2a	2.38	2.38	2.35	2.35
2b	2.50	2.50	2.41	2.43
3	3.97	3.97	4.11	4.14
3OH	5.12	5.12	5.10	-
6	3.07	3.07	3.08	3.10
7	3.49	3.49	3.48	3.51
7OH	4.46	4.46	3.18	-
8	1.34	1.34	1.35	1.38
9a	1.15	1.15	1.03	1.05
9b	1.40	1.40	1.55	1.56
10a	1.15	1.15	1.15	1.19
10b	1.46	1.35	1.35	1.37
11a	1.35	1.90	1.88	1.90
11b	1.66	2.18	2.21	2.22
12	2.84	5.38	5.44	5.48
13	3.06	5.44	5.39	5.40
14a	1.76	2.35	2.15	2.14
14b	2.10	2.70	2.70	2.71
15	5.27	5.27	5.12	5.10
17	6.50	6.50	6.50	6.52
19	7.35	7.35	7.33	7.34
21	2.65	2.65	2.65	2.67
22	0.94	0.94	0.91	0.93
23	1.21	1.21	1.20	1.21
24	1.06	1.06	1.06	1.06
25	0.90	0.90	0.89	0.88
26	-	-	-	-
27	2.10	2.10	2.12	2.14

Refences and comments:

1. G. Höfle et al. Angew. Chem. Int. Ed. Engl. 1996, 35, 1567-1569.
2. Spectrum taken Nov. 15, 1996 from a sample of epothilone C isolated Oct./Nov. 1996.
3. Opposition Statement, p. 54-55.

Conclusion:

Chemical shifts for C1-C8 and C15-C27 in EP-B-1186606 are identical with epothilone A, those for C9-C14 are identical with epothilone C.

Tab. 4 ^{1}H-NMR chemical shifts of epothilone D in DMSO-D_6

H-Atoms	Epo B[1]	EP-B-1186606 18.11.96	GBF[3] 15.11.96	Kosan[5]
2a	2.35	2.35	2.32	2.34
2b	2.38	2.38	2.37	2.34
3	4.10	4.10	4.15	4.14
3OH	5.08	5.08	5.10	-
6	3.11	3.11	3.09	3.09
7	3.48	3.48	3.49	3.48
7OH	4.46	4.46	3.18	-
8	1.29	1.29	1.34	1.33
9a	1.14	1.14	1.15	1.15
9b	1.38	1.38	1.35	1.35
10a	1.14	1.14	1.02	1.02
10b	1.43	1.35	1.65	1.65
11a	1.31	1.75	1.76	1.75
11b	1.61	2.10	2.30	2.29
12	-	-	-	-
13	2.84	5.08	5.10	5.14
14a	2.05	2.30	2.12	2.12
14b	1.84	2.65	2.66	2.66
15	5.29	5.29	5.10	5.09
17	6.51	6.51	6.48	6.48
19	7.35	7.35	7.33	7.33
21	2.65	2.65	2.65	2.65
22	0.90	0.90	0.90	0.90
23	1.19	1.19	1.18	1.18
24	1.07	1.07	1.08	1.08
25	0.91	0.91	0.91	0.91
26	1.19	1.63	1.64	1.64
27	2.11	2.11	2.11	2.11

Refences and comments:

1. G. Höfle et al. Angew. Chem. Int. Ed. Engl. 1996, 35, 1567-1569.
2. Spectrum taken Nov. 15, 1969 from a sample of epothilone D isolated Oct./Nov. 1996.
3. Recent sample of epothilone D.
4. Opposition Statement, p. 58-59.

Conclusion:

Chemical shifts for C1-C8 and C15-C27 in EP-B-1186606 are identical with epothilone A, those for C9-C14 are identical with epothilone C.

Tab. 5 ^{13}C-NMR chemical shifts of epothilone isomers (Epos) with molecular mass m/z = 477 in $CDCl_3$

Nr.	Epo C Hardt [1]	trans-Epo C Schinzer [2]	trans-Epo C Danishefsky[3]	Epo D_1 Hardt [1]	Epo D_2 Hardt [1]	Max. delta > 2.0
1	220.6	219.9	219.9	217.0	216.8	3.6
2	170.4	170.5	170.5	169,7	170.4	
3	165.0	164.9	165.0	165.0	165.9	
4	152.1	152.1	152.0	152.2	152.3	
5	138.7	137.1	137.1	138.5	139.8	
6	133.5	134.3	134.4	137.7	137.5	4.2
7	125.0	125.7	125.7	120.7	120.5	4.8
8	119.5	119.8	119.8	121.1	119.2	
9	115.8	116.0	116.0	116.3	116.3	
10	78.5	76.6	77.6	78.8	80.8	2.3
11	74.2	75.8	75.8	77.2	74.3	3.0
12	72.4	72.4	72.4	67.7	69.7	4.7
13	53.4	52.5	52.5	52.5	48.6	4.8
14	41.8	43.6	43.6	46.5	48.4	6.6
15	39.3	38.9	38.8	30.6	39.9	
16	38.6	37.7	37.8	37.6	36.6	
17	31.8	36.2	36.2	32.3	32.7	4.4
18	31.5	32.4	32.5	31.8	32.2	
19	27.6	30.5	30.6	29.5	30.9	3.3
20	27.5	27.2	27.3	25.5	26.0	
21	22.7	21.0	21.0	22.1	23.6	
22	19.1	20.7	20.7	19.2	19.2	
23	18.7	19.1	19.0	16.6	17.1	2.1
24	15.9	16.4	16.4	15.5	15.4	
25	15.5	15.7	15.7	14.5	12.7	2.8
26	13.5	14.8	14.8	9.7	12.4	3.8

Refences and comments:

5. I. H. Hardt et al., J. Nat. Prod. **2001**, *64*, 847- 856.
6. D. Schinzer et al., Chem. Eur. J. **1999**, *5*, 2483- 2491.
3. PCT/US97/22381; D. Meng et al., J. Am Chem. Soc. **1997**, *119*, 10073- 10092.

Connclusion:

Chemical shifts for individual carbon atoms vary by 2.1 up to 6.6 ppm.

Tab. 6 ^{13}C-NMR chemical shifts of epothilone C in $CDCl_3$

Nr.	Danishefsky Original [1,2]	Danishefsky Corrected [3]	Nicolaou [4]	Nicolaou [5]	Schinzer [6]	Hardt [7]	Max. delta
1	226.5	220.4	220.6	220.2	220.5	220.6	0.4
2	176.5	170.4	170.4	170.6	170.3	170.4	0.3
3	171.1	165.0	165.0	165.4	165.0	165.0	0.4
4	158.2	152.1	151.9	153.8	152.0	152.1	1.8
5	144.7	138.6	138.7	139.2	138.6	138.7	0.6
6	139.6	133.5	133.4	134.1	133.4	133.5	0.7
7	131.1	125.0	125.0	126.1	125.0	125.0	1.1
8	125.7	119.6	119.4	120.4	119.5	119.5	1.0
9	122.0	115.9	115.8	116.9	115.8	115.8	0.8
10	84.6	78.5	78.4	79.2	78.4	78.5	0.8
11	80.2	74.1	74.1	74.9	74.1	74.2	0.8
12	78.6	72.5	72.3	73.2	72.4	72.4	0.9
13	59.4	53.3	53.3	54.2	53.3	53.4	0.9
14	47.9	41.8	41.7	42.5	41.8	41.8	0.8
15	45.4	39.3	39.2	40.3	39.2	39.3	1.1
16	44.6	38.5	38.5	39.5	38.5	38.6	1.0
17	38.5	32.4	32.4	32.9	32.5	31.8	1.1
18	37.9	31.8	31.7	32.6	31.7	31.5	1.1
19	33.7	27.6	27.6	28.6	27.6	27.6	1.0
20	33.6	27.5	27.4	28.4	27.5	27.5	1.0
21	28.7	22.6	22.7	23.3	22.7	22.7	0.7
22	25.1	19.0	19.0	19.3	19.0	19.1	0.3
23	25.0	18.9	18.6	19.1	18.7	18.7	0.5
24	21.9	15.8	15.9	16.4	15.8	15.9	0.6
25	21.7	15.6	15.5	16.3	15.5	15.5	0.8
26	19.6	13.5	13.5	14.4	13.5	13.5	0.9

Refences and comments:

1. PCT/US97/22381
2. D. Meng et al., J. Am Chem. Soc. **1997**, *119*, 10073- 10092.
3. Offset of 6.1 ppm.
4. K. C. Nicolaou et al., J. Amer. Chem. Soc. *119*, 7960, **1997**.
5. K. C. Nicolaou et al., J. Amer. Chem. Soc. *119*, 7974, **1997**.
6. D. Schinzer et al., Chem. Eur. J. **1999**, *5*, 2483- 2491.
7. I. H. Hardt et al., J. Nat. Prod. **2001**, *64*, 847- 856.

Conclusion:

Chemical shifts for individual carbon atoms vary by 0.3 up to 1.1 ppm.

Tab. 7 ^{1}H- and ^{13}C-NMR chemical shifts of epothilones C and D in DMSO-D_6

	Epothilone C		Epothilone D	
H - Atoms	GBF[1] 1. 9. 05	Kosan[2]	GBF[1] 1. 9. 05	Kosan[2]
2a	2.34	2.35	2.34	2.34
2b	2.41	2.43	2.37	2.34
3	4.11	4.14	4.14	4.14
3OH	5.10	-	5.08	-
6	3.08	3.10	3.09	3.09
7	3.49	3.51	3.48	3.48
7OH	-	-	4.41	-
8	1.36	1.38	1.34	1.33
9a	1.03	1.05	1.15	1.15
9b	1.55	1.56	1.35	1.35
10a	1.16	1.19	1.01	1.02
10b	1.36	1.37	1.66	1.65
11a	1.89	1.90	1.76	1.75
11b	2.21	2.22	2.30	2.29
12	5.47	5.48	-	-
13	5.38	5.40	5.15	5.14
14a	2.15	2.14	2.12	2.12
14b	2.69	2.71	2.66	2.66
15	5.13	5.10	5.09	5.09
17	6.50	6.52	6.48	6.48
19	7.33	7.34	7.34	7.33
21	2.65	2.67	2.66	2.65
22	0.91	0.93	0.90	0.90
23	1.19	1.21	1.18	1.18
24	1.06	1.06	1.08	1.08
25	0.89	0.88	0.91	0.91
26	-	-	1.64	1.64
27	2.12	2.14	2.11	2.11

	Epothilone C		Epothilone D	
C - Atoms	GBF[1] 1. 9. 05	Kosan[2]	GBF[1] 1. 9. 05	Kosan[2]
1	170.08	170.6	170.08	170.3
2	38.77	38.8	38.88	39.1
3	70.85	70.8	70.81	71.0
4	53.18	53.2	53.27	53.5
5	217.50	217.5	217.41	217.6
6	44.28	44.3	44.46	44.7
7	75.13	75.1	75.45	75.6
8	36.53	36.6	36.29	36.5
9	27.57	27.6	29.91	30.1
10	29.98	30.0	25.91	26.1
11	27.57	27.6	31.57	31.8
12	133.08	133.1	138.38	138.6
13	124.54	124.5	120.28	120.5
14	31.05	31.1	31.86	32.1
15	78.44	78.4	78.94	79.1
16	137.35	137.3	137.57	137.8
17	118.72	118.7	118.78	119.0
18	152.25	152.3	152.24	152.5
19	117.48	117.5	117.46	117.7
20	164.20	164.2	164.19	164.4
21	18.86	18.9	18.85	19.1
22	20.22	20.2	19.93	20.1
23	22.49	22.5	22.54	22.7
24	16.01	16.0	16.24	16.4
25	17.37	17.5	17.71	17.9
26	-	-	22.95	23.2
27	14.65	14.7	14.52	14.7

Refences and comments:

1 New isolates from So ce90wild DSM 6773 (produced August 1 – 31, 2005).
2 Opposition Statement, p. 54-55.

Conclusion: All signals for GBF and Kosan samples are identical within the experimental error. The maximal shift differences of 0.52 and 0.22 ppm are observed for C-1.